# Nitrogen Metabolism in *Corynebacterium glutamicum* ATCC 13032

Anton A. Schulz



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*"Look not upon a man in his hour of triumph*
*And say it was luck that has brought this.*
*For no man can see or anyone guess*
*The agony by which it was achieved*
*While other men slept"*

*Anon.*

# Contents

# Abbreviations

| | |
|---|---|
| ADP | adenosine 5' diphosphate |
| AMP | adenosine 5' monophosphate |
| Ap | ampicillin |
| ATase | adenylyltransferase |
| ATP | adenosine 5' triphosphate |
| bp | base pairs |
| CAT | chloramphenicol acetyltransferase |
| CFE | cell free extract |
| Cm | chloramphenicol |
| CRP | catabolite repressor protein |
| DIG | digoxigenin-11-dUTP |
| dNTPs | deoxyribonucleotide triphosphates |
| EDTA | ethylene diamine tetra-acetate |
| FAD | flavin adenine dinucleotide |
| FMN | flavin mononucleotide |
| GDH | glutamate dehydrogenase |
| GMP | 5'-guanylic acid |
| GOGAT | glutamate synthase |
| GS | glutamine synthetase |
| γ-GT | γ-glutamyl transferase |
| h | hour(s) |
| IMP | 5'-monophosphate 5'-inosinic acid |
| kb | kilobases |
| kDA | kiloDalton |
| Km | kanamycin |
| LA | Luria agar |
| LB | Luria-Bertani broth |
| min | minute(s) |
| MIC | minimum inhibitory concentration |
| MCS | multiple cloning site |
| MM | minimal medium |
| NAD | nicotinamide adenine dinucleotide |
| Nx | nalidixic acid |
| ORF | open reading frame |
| PCR | polymerase chain reaction |
| PTS | phosphotransferase system |
| rpm | revolutions per minute |
| RT | room temperature |
| s | second(s) |
| SDS | sodium dodecyl sulfate |
| SVPD | snake venom phosphodiesterase |
| UTase/UR | uridylyltransferase/uridylyl-removing enzyme |
| XMP | 5'-xanthylic acid |
| YT | yeast tryptone medium |

# Summary

*Corynebacterium glutamicum* is extensively used for the commercial production of a host of amino acids including lysine, glutamate, and threonine. Consequently, much research has been directed at analyzing nitrogen metabolism in this bacterium. In particular, our research focused on investigating the regulation of nitrogen assimilation. Initially, we searched for homologs of the *Streptomyces glnR*, *glnII*, and *glnE* genes in *C. glutamicum*. These studies, however, were met with limited success, and we therefore decided to use promoter probe vectors in order to identify nitrogen-responsive promoters. We isolated several promoters that were induced under low nitrogen. Some of these sequences belonged to genes that were involved in nitrogen metabolism, including *glnA*, but no novel nitrogen regulatory genes were identified.

As is the case in many bacterial systems, glutamine synthetase (GS) and glutamate synthase (GOGAT) activities in *C. glutamicum* were dependent on nitrogen status. Enzyme assays showed that the activities of both enzymes were significantly increased during nitrogen limitation. Similarly, transcription of their encoding genes, *glnA* and *gltBD*, respectively, was also induced under these conditions. Experiments with snake venom phosphodiesterase (SVPD) indicated the significance of posttranslational modification in the control of GS activity. In parallel with these studies, we investigated the effect of glucose (carbon) status on the activities of these enzymes and transcription of their encoding genes. Low glucose concentrations resulted in reduced enzyme activities, while high glucose stimulated *glnA* but not *gltBD* transcription. These studies showed that there is a definite link between nitrogen regulation and carbon metabolism in *C. glutamicum*. Primer extension analysis identified the start of transcription of *glnA*, and the promoter structure conforms to that of typical *C. glutamicum* promoters.

Having established the effect of nitrogen status on GS and GOGAT, we proceeded to generate *C. glutamicum* disruption mutants of *glnA*. These mutants displayed no detectable GS activity, and were unable to grow in minimal medium unless glutamine was supplied. Sequence analysis of the *C. glutamicum gltBD* region revealed the existence of an upstream open reading frame, which we designated *hkm*, that may

encode a sensor protein. The putative Hkm protein contains a conserved PAS domain which is a motif involved in signal transmission in proteins that sense a variety of conditions. In order to characterize this gene, we generated *C. glutamicum hkm* disruption mutants via homologous recombination. The mutants were unaffected in their GS activity profiles and grew well on various nitrogen sources. In contrast, GOGAT assays demonstrated that the *hkm* mutants had reduced induction of activity in response to nitrogen starvation in comparison to the wild type. These results were confirmed by RNA analyses that showed reduced transcription of *gltBD* under the same condition. Promoter activity studies revealed that *hkm* is most likely transcribed at a low, constitutive level. The occurrence of a PAS domain in the putative Hkm protein and the effect of *hkm* deletion on GOGAT expression suggest an involvement of Hkm in sensing nitrogen status and controlling GOGAT activity. Amongst other possibilities, we propose a putative interaction of Hkm with the *C. glutamicum* global nitrogen repressor, AmtR.

# Chapter 1

## General Introduction

### 1.1 Introduction to *Corynebacterium glutamicum*

*Corynebacterium glutamicum* is an aerobic, Gram-positive soil bacterium that belongs to the group of mycolic acid containing actinomycetes (Stackebrandt *et al.*, 1997). Although this bacterium is often rod-shaped (Fig. 1.1), the cells generally have an irregular morphology ("coryneform") and may even be club-shaped at times (Collins and Cummins, 1986). While *C. glutamicum* itself is non-pathogenic, other members of the genus, such as *Corynebacterium diptheria*, are associated with a number of human diseases (Cummins *et al.*, 1974). Indeed, the phylogenetic relatives of *C. glutamicum* include pathogens such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*.



**Fig 1.1** Phase contrast microphotograph of *C. glutamicum*. Bar = 10μm. Taken from Liebl (2002).

A typical characteristic of members of the genus *Corynebacterium* is the relatively high GC content of their DNA, where the base composition varies from 46 to 71 mol% G+C. However, most species within the genus have DNA with between 51 and 68 mol% G+C (Goodfellow and Minnikin, 1981). Members of *Corynebacterium* and closely related genera have a cell wall structure that differs markedly form that of other Gram-positive bacteria (Liebl, 2002).

The cell wall structure of *Corynebacterium* contains directly-linked peptidoglycan type A1γ (Schleifer and Kandler, 1972) with *meso*-diaminopimelic acid as the crosslinking amino acid, and the predominant cell wall sugars are galactose and arabinose. Mycolic acids are also present (except in *C. amycolatum*), as well as straight-chain saturated or monounsaturated fatty acids, 10-methyl-branched chain acids (in some cases), and eight and/or nine isoprene unit dihydrogenated menaquinones.

### 1.1.1 Taxonomy of *Corynebacterium*

Corynebacterial nomenclature and taxonomy has been the subject of great dispute since the genus *Corynebacterium* was first proposed by Lehmann and Neumann in 1896. Initially, the genus was essentially defined in morphological terms to include Gram-positive, non-acid-fast, non-sporeforming, nonmotile, nonbranching, pleomorphic bacteria (Goodfellow and Minnikin, 1981). Consequently, a vast array of poorly-defined Gram-positive bacteria that are not strictly coryneform were classified in the family Corynebacteriaceae in the sixth and seventh edition of "Bergey's Manual of Determinative Bacteriology" (Breed *et al.*, 1948; Breed *et al.*, 1957). This included the genera *Listeria* and *Erysipelothrix*, and was further extended to include *Jensenia*, *Mycobacterium* and *Nocardia* (Davis and Newton, 1969). In the eighth edition of Bergey's Manual (Rogosa *et* al., 1974), the genera *Cellulomonas*, *Corynebacterium*, *Arthrobacter*, and *Kurthia* (tentatively) were also classified in the section entitled "Coryneform Group of Bacteria". According to this system, *Brevibacterium* and *Microbacterium* were listed as genera *incertae sedis*. It seems that at one time or another the term coryneform has been applied to a myriad of genera including *Arachnia, Actinomycyes, Rothia, Oerskovia, Bifidobacterium, Bacterionema, Eubacterium, Rhodoccoccus,* and *Propionibacterium* (Goodfellow and Minnikin, 1981). Clearly, bacterial classification based primarily on morphology was not definitive, and the term "coryneform" was used rather subjectively.

Other chemotaxonomic studies comparing cell wall composition and lipid profiles indicated that members of the genera *Nocardia*, *Rhodococcus* and *Mycobacterium* are close relatives of *Corynebacterium*, and these four genera were then combined into the "CMN-group" (Barksdale, 1970). Several years later, these genera were placed together

with *Caseobacter* (later transferred to *Corynebacterium*) into a group called "Mycobacteriaceae" (Bousfield and Goodfellow, 1976). A different classification system considered *Corynebacterium* and *Mycobacterium* separately, and grouped the other mycolate-containing, cell wall type IV actinomycetes into the family Nocardiaceae (Goodfellow, 1992). Unlike previous morphology-based classifications, this system was also consistent with rigorous phylogenetic analysis, i.e. 16S rDNA sequence comparison. It then became clear that the CMN-group, which we today consider as including the genera *Dietzia, Tsukamurella, Corynebacterium, Nocardia, Gordona, Mycobacterium, Rhodococcus* and the mycolate-less *Turicella*, forms a robust monophyletic taxon (Liebl, 2002).

In 1997, Stackebrandt proposed a new hierarchal classification system for the actinomycete subphylum, which is based solely on 16S rRNA/rDNA sequence data. According to this classification system, the genera *Corynebacterium* and *Turicella* form the family Corynebacteriaceae, which is one of six families within the suborder Corynebacterinae which is within the order Actinomycetales. In addition to Corynebacteriaceae, the suborder comprises Dietziaceae, Gordoniaceae, Mycobacteriaceae, Tsukamurellaceae, and Nocardiaceae. The phylogenetic tree of members of the suborder Corynebacerinae and genus *Corynebacterium* in particular is show in Fig. 1.2. For indepth reviews regarding the history as well as current taxonomic classification of *Corynebacterium* see Goodfellow and Minnikin (1981) and Stackebrandt *et al.* (1997).



While *Corynebacterium* has now been firmly placed in terms of phylogenetic lineage, it is important to note that this genus is phenotypically diverse and contains both aerobes and facultative anaerobes, both mycolate- and non-mycolate containing species, and both pathogenic and nonpathogenic species (Liebl, 2002). Over 50 species of *Corynebacterium* have now been identified, over half of them in the last decade.

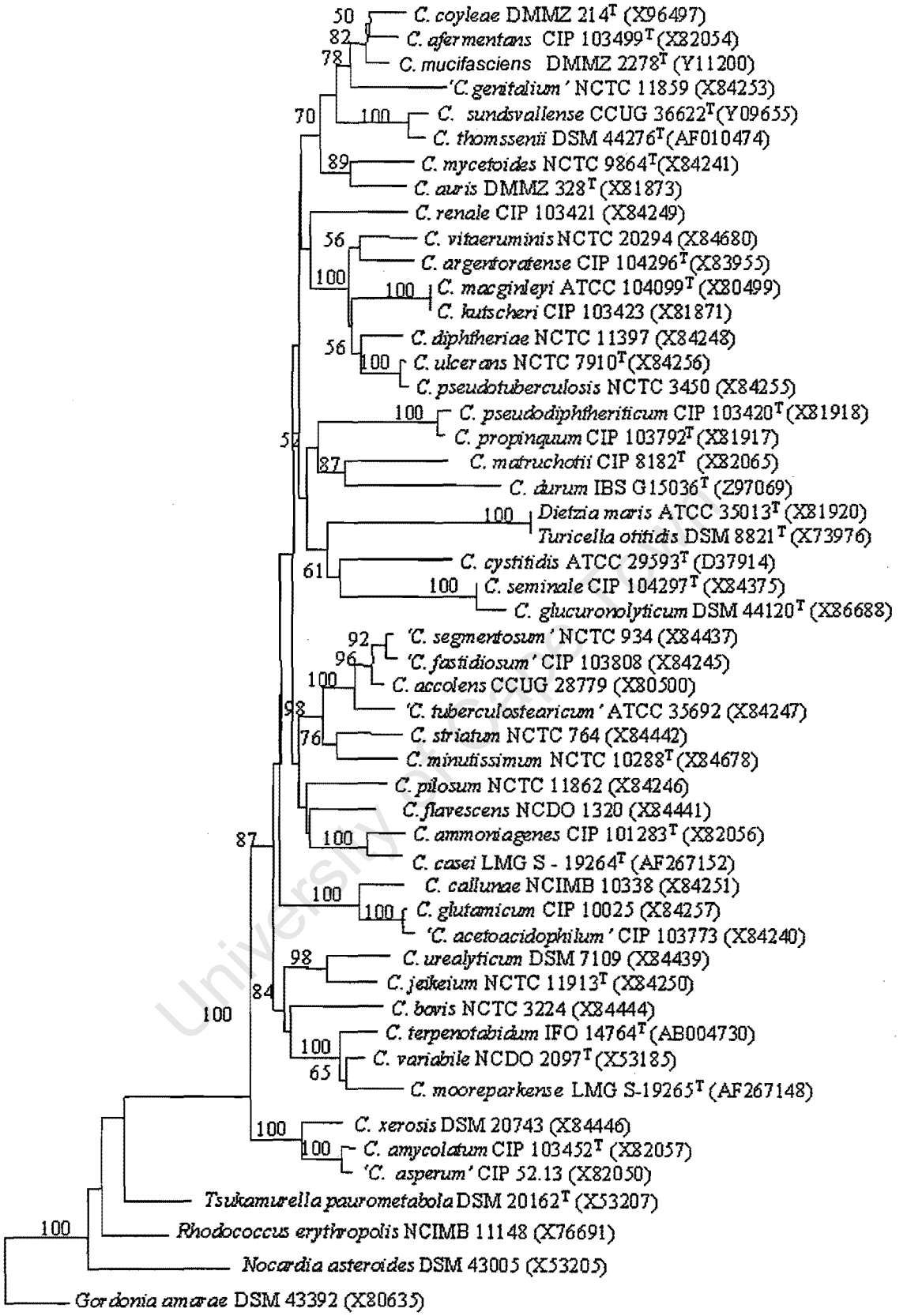



**Fig. 1.2** Unrooted tree of the phylogenetic relationships of members of *Corynebacterium* and their close relatives. The tree is based on 16S rRNA data and was constructed by the neighbour-joining method. Bootstrap values (indicated at the branch points) are expressed as percentages of 1000 replications. (Taken from Liebl, 2002).

Some species of *Corynebacterium* have been more extensively studied than others. The primary reason why *C. glutamicum* and related strains are so well-characterised, is because they are used for the fermentative production of a host of amino acids, including lysine, leucine, threonine, glutamate and aspartate (Patek *et al.,* 1994; Malumbres and Martín, 1996; Leuchtenberger, 1996).

## 1.2 *C. glutamicum* and Amino Acid Fermentation

Coryneform bacteria have long been used in industrial fermentations that produce amino acids (Kinoshito, 1985). Selected strains of *C. glutamicum* and closely-related species are employed in large-scale fermentation processes, and supply the world market with over $10^9$ kg of amino acids per annum. Two of the most significant of these are L-lysine and L-glutamate (Leuchtenberger, 1996). L-lysine is an important animal feed supplement, while L-glutamate is used for the production of the food flavour enhancer monosodium glutamate or MSG (Malumbres and Martín, 1996). South Africa also contributes to international amino acid production, and AECI, a major South African chemical company, scaled-up its commercial production in the mid-1990's by establishing a large lysine production plant on the outskirts of Durban, Kwazulu-Natal, South Africa. Indeed, the research described here was, in part, funded by the research and development division of AECI.



*C. glutamicum* was first isolated in 1957 in the course of screening for efficient glutamate producers (Kinoshito, 1985). It was subsequently found that under conditions of biotin limitation, *C. glutamicum* excretes large amounts of glutamate into the growth medium (more than 100g/L). In fact, in a typical fermentation, the molar yield of glutamate from sugar is in the range 0.5 to 0.75 (Demain, 2000). Initial theories regarding glutamate excretion by *C. glutamicum* suggested leakiness of the cells due to membrane permeability (Kimura, 1963; Shiio *et al.*, 1963). It was also proposed that glutamate excretion is achieved by the reverse action of the glutamate uptake system (Clement *et al.*, 1986). Furthermore, it was initially thought that the absence of the enzyme α-ketoglutarate dehydrogenase was also a contributing factor in glutamate excretion. This theory was abandoned when it was later discovered that this enzyme was indeed present in *C. glutamicum* (Shiio and Ujigawa-Takeda, 1980). Subsequent

research, however, has shown that a specific, energy-dependent transport system is responsible for this glutamate excretion during biotin limitation (Hoischen and Krämer, 1989; Gutman *et al.*, 1992).

Since industrial fermentations often utilise cheap, biotin-rich substrates (such as cane molasses), alternative, biotin-independent processes had to be developed in order to harvest glutamate (Liebl, 2002). One approach involved the addition of penicillin during logarithmic growth (Kinoshito, 1985). Although high yields were achieved by this method, cell viability dropped rapidly. Alternative methods included the addition of fatty acid derivatives such as Tween 20 or the use of glycerol-requiring auxotrophs grown under glycerol starvation (Kinoshito and Nakayama, 1978). In small-scale fermentations, high yields of glutamine could be achieved by the addition of amine surfactants (Dupperay *et al.*, 1992) or the antibiotic tetracaine (Krämer, 1994).

Although the commercial demand for glutamate is high, lysine is still considered the most economically important amino acid produced by coryneform bacteria (Leuchtenberger, 1996). While the industrial molar yields of lysine are generally lower than those obtained for glutamate, the lysine concentration can reach up to 170 g/L in the culture medium (Demain, 2000). In contrast to glutamate production, the high-yield fermentation of lysine by *C. glutamicum* cannot simply be achieved by adjusting culture conditions and genetically modified strains are required (Cremer *et al.*, 1988). In fact, no wild-type strains of *C. glutamicum* secrete lysine (Cremer *et al.*, 1991). Originally, classical mutation and selection techniques were used successfully in the development of many strains that overproduce and excrete amino acids (Malumbres and Martín, 1996). However, with the advent of recombinant DNA technology and transformation systems, the activity of specific enzymes in *C. glutamicum* could be altered and tightly regulated (Cremer *et al.*, 1991). The ultimate goal of these techniques was to direct the flow of precursors along a defined route to maximize the rate and conversion into amino acids. However, simply amplifying the genes involved in amino acid biosynthesis does not always result in increased yields. This is because such gene amplification often redirects carbon flux distribution at key branch points from the normal distribution associated with balanced growth (Stephanopoulos and Vallino, 1991). In fact, it seems

that flux distributions that result in the highest amino acid yield are usually significantly divergent from wild-type flux distribution.

A major factor in lysine over-production by *C. glutamicum* is the lack of regulation of the lysine-specific biosynthetic enzymes within the aspartate family (Yeh *et al.*, 1998). Lysine, together with threonine, methionine and isoleucine, belongs to the aspartate family of amino acids, and, as seen in Fig. 1.3, several enzymatic steps lead to the synthesis of these amino acids. The only point of regulation for the lysine-specific enzymes occurs at aspartokinase, the first enzyme in the sequence for the synthesis of the aspartate family amino acids. This is in contrast to other bacterial systems, such as that of *E. coli*, which has three different aspartokinase isoenzymes (Cohen, 1983). Two of these aspartokinases are bifunctional proteins that also display homoserine dehydrogenase activity, and each of these aspartokinases is regulated by a different end product. In *C. glutamicum*, the single aspartokinase enzyme is subject to concerted feedback inhibition by threonine and lysine (Fig. 1.3) (Malumbres and Martín, 1996). Similarly, the flow from aspartate-β-semialdehyde to either homoserine or the lysine pathway (see Fig. 1) is regulated solely at the level of homoserine dehydrogenase. Homoserine dehydrogenase activity is repressed by methionine and partly by isoleucine (Cremer *et al.*, 1988).

Strains have been engineered where carbon flux has been redirected at branch points by disrupting the genes involved in pathways that compete with lysine or threonine production. For example, mutations in isopropylmalate dehydratase, an enzyme involved in leucine biosynthesis, result in a $Leu^-$ phenotype and lysine overproduction (Patek *et al.*, 1994). During the course of screening for efficient amino acid-producing mutants of *C. glutamicum*, Kinoshito and coworkers (1958) isolated strains that excreted lysine due to mutations in homoserine dehydrogenase. These strains were auxotrophic for both methionine and threonine. Other lysine-producing strains include regulatory mutants that display resistance to feedback inhibition. The isolation of *C. glutamicum* mutants resistant to the lysine analogs S-2-aminoethyl-L-cysteine or O-2-aminoethyl-L-serine lead to the discovery of strains with feedback-resistant aspartokinase enzymes that over-produce lysine (Sano and Shiio, 1970). The *C.*

*glutamicum* strains currently employed in industrial fermentations are usually a combination of both auxotrophic and regulatory mutations (Leuchtenberger, 1996).

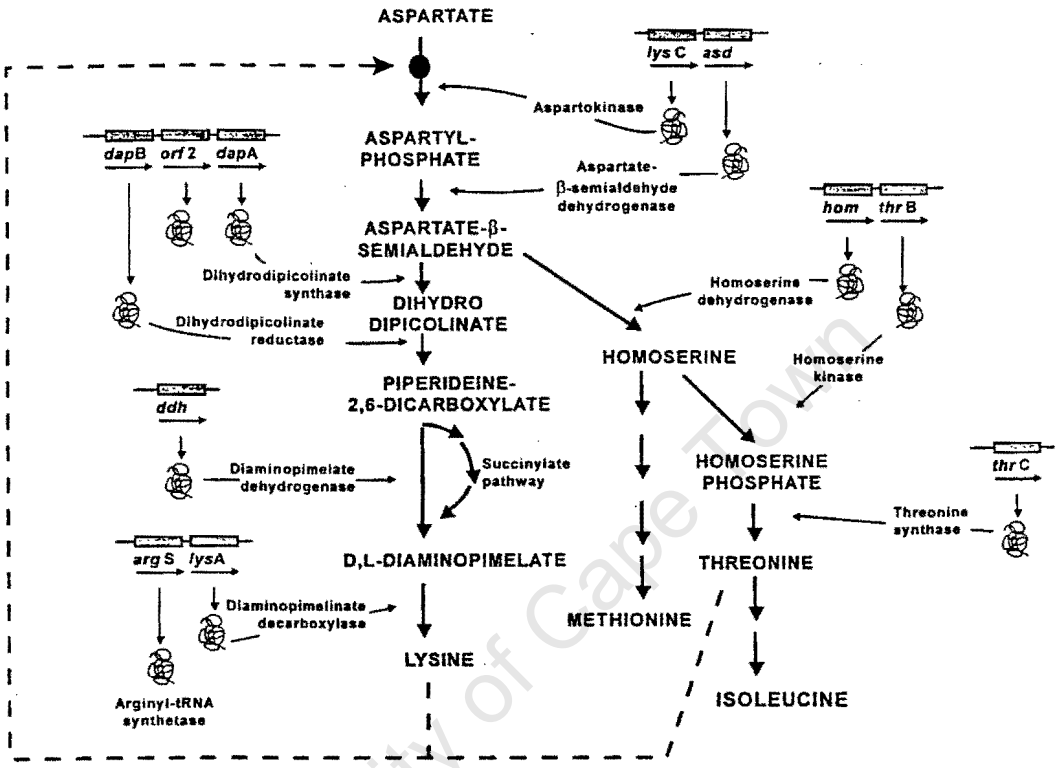



**Fig 1.3** Organization and genetic control of lysine and threonine biosynthesis from aspartate in *Corynebacterium*. Concerted feedback inhibition is indicated by the dashed line (taken from Malumbres and Martín, 1996).



By similar genetic engineering, the various other amino acid biosynthetic genes can be preferentially expressed to yield the desired product in high concentrations. Some more recent, advanced techniques that are useful for studying amino acid metabolism include mathematical modeling, metabolic flux analysis (using either *in vivo*-NMR or MALDI-TOF mass spectrometry)(Tesch *et al.*, 1999; Hermann *et al.*, 2001), DNA chip technology (Loos *et al.*, 2001) and proteome analysis (Hermann *et al.*, 2001). These modern analytical techniques in combination with genetic engineering are likely to result in further improved amino acid-producing strains (De Graaf *et al.*, 2001).

## 1.3 Other Industrial Applications of *Corynebacterium*

Nucleotides and other metabolites are also produced by industrial fermentations of *Corynebacterium*, although on a far smaller scale than amino acids (Komata, 1976). Selected mutant strains are used to produce a number of food flavour-enhancers including the ribonucleoside 5'-monophosphate 5'-inosinic acid (IMP), 5'-xanthylic acid (XMP), and 5'-guanylic acid (GMP)(Demain, 1978). Wild-type *Corynebacterium ammoniagenes* is able to convert hypoxanthine to IMP, guanine to GMP, GDP and GTP, and adenine to AMP, ADP, and ATP by what are known as "salvage reactions" (Liebl, 2002). In the case of IMP production, the concentration of the manganese ions is an important factor affecting yields (Furuya *et al.*, 1970). When $Mn^{2+}$ concentrations are sufficient, nucleotide production can be achieved by the addition of certain antibiotics or surfactants to the medium, or the use of mutant *C. ammoniagenes* strains that accumulate IMP under conditions of $Mn^{2+}$ excess (Furuya *et al.*, 1969). An alternative method of IMP synthesis involves the fermentative production of inosine by *C. ammoniagenes* mutants, followed by chemical phosphorylation of the nucleoside by phosphoryl chloride. A more recent method of IMP production involves conventional inosine fermentation by *C. ammoniagenes* followed by enzymatic phosphorylation using engineered guanosine/ inosine kinase-overexpressing *E. coli* cells (Mori *et al.*, 1997).

*C. ammoniagenes* has also been used to produce nicotinamide adenine dinucleotide (NAD) when grown in medium which contains adenine and nicotinic acid or nicotinamide (Liebl, 2002). More recently, Koizumi and colleagues (2000) reported high yields of riboflavin (vitamin $B_2$) by an engineered strain of *C. ammoniagenes* that has enhanced activities of the riboflavin biosynthetic enzymes.

In both small- and industrial scale cultures of *C. glutamicum*, nitrogen availability is a critical determinant of amino acid as well as other product yields. Since nitrogen metabolism and amino acid synthesis in particular are inextricably linked, a detailed understanding of nitrogen assimilation is essential if further industrial strain improvements are to be carried out. In this respect, extensive research during the past

decade and the advent of specialised vectors and molecular techniques have greatly aided elucidation of the relevant nitrogen pathways in *C. glutamicum*.

## 1.4 Bacterial Nitrogen Assimilation

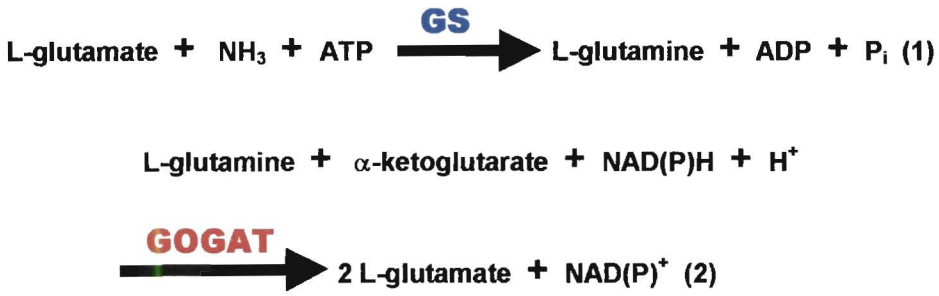
### 1.4.1 Nitrogen sources

When bacteria are grown in a simple salts medium containing ammonia and glucose, the organisms are required to synthesize all amino acids and nitrogen-containing compounds from ammonia and intermediate metabolites (Schreier, 1993). In addition to ammonia, bacteria are also able to utilise a variety of nitrogenous compounds as sources of cellular nitrogen (Reitzer and Magasanik, 1987). These compounds may be inorganic in nature such as nitrate or diatomic nitrogen, or organic such as amino acids, urea, amino sugars and nucleosides. In general, ammonia is the preferred nitrogen source for bacterial growth since it supports a higher growth rate than any other nitrogen source. However, in certain bacteria other nitrogen sources are preferred over ammonia. In *Bacillus subtilis*, for example, glutamine is the preferred nitrogen source (Atkinson and Fisher, 1991). The selective utilisation of certain nitrogenous compounds over others depends on the available uptake systems and active enzymatic pathways as well as the regulation of these systems.

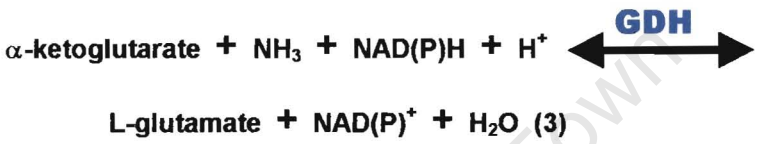


### 1.4.2 Glutamine and glutamate biosynthesis

In most organisms that have been studied, glutamate formation seems to be the primary route for nitrogen assimilation (Ertan, 1992b). Irrespective of nitrogen source, bacteria absolutely require ammonia for glutamine synthesis, and all nitrogen sources must be catabolised to yield ammonia (Reitzer, 1998). The subsequent assimilation of ammonia involves the action of three enzymes, namely glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH). GS and GOGAT catalyze the formation of glutamine and glutamate, respectively, in the following reactions:




GDH, on the other hand, is able to catalyze the reversible reductive amination of α-ketoglutarate in the following reaction:




The GS/GOGAT and GDH pathways seem to be ubiquitous in bacteria, although the contribution of each to the synthesis of glutamate may differ (Merrick and Edwards, 1995). For example, in microorganims such as *Clostridium pasteurianum* (Dainty, 1972) and *Streptomyces clavuligerus* (Brana *at al.* 1986), ammonia assimilation occurs exclusively via the GS/GOGAT pathway. In contrast, GDH appears to be the sole ammonia assimilatory enzyme in the ruminal bacterium *Streptococcus bovis* (Chen and Russell, 1989).

When bacteria are grown in minimal medium with an ample supply of ammonia, the nitrogen for all other nitrogen-containing compounds is derived almost exclusively from glutamate and glutamine (Reitzer and Magasanik, 1987). Glutamate provides the α-amino groups required for the synthesis of all the amino acids, and half the nitrogen for the pyrimidine, purine, and imadazole rings. Glutamine, on the other hand, is a nitrogen donor for the synthesis of amino sugars, ρ-aminobenzoate, and NAD, as well as the remaining nitrogen required for purines, pyrimidines, histidine and tryptophan (Wohlheuter *et al.*, 1973). It is estimated that 1kg dry weight of *E. coli* contains up to 12g of nitrogen atoms. As seen in reaction (1) above, cells absolutely require ammonia for the synthesis of glutamine, and, depending on the medium, may require ammonia for the synthesis of all other nitrogen-containing compounds. This requirement depends

on the nature and concentration of the nitrogen source as well as the products of its catabolism. If the ammonia concentration is sufficiently high, the compound is assimilated into glutamine and glutamate by GS and GOGAT, respectively (Helling, 1994). For nitrogen sources whose degradation produces glutamate, such as aspartate, proline and γ-aminobutyrate, ammonia is needed only for glutamine production (Reitzer, 1998). For nitrogen sources such as serine, which generate ammonia but not glutamate, the ammonia channeled into glutamine provides the nitrogen required for the synthesis of glutamate by GOGAT. Therefore, the GS/GOGAT pathways form an energy-dependent ammonia assimilatory cycle such that all cellular nitrogen is derived from ammonia and the amide group of glutamine. The requirement for glutamate can therefore be reduced but not eliminated.

### 1.4.3 Glutamine synthetase

#### *1.4.3.1 Classes of GS enzymes*

Clearly, the synthesis of glutamine by the action of GS is a major branch point in both nitrogen and cellular metabolism. As a result, the GS enzyme has been extensively studied and characterised in a large number of bacterial species (Backman *et al*, 1981; Barros *et al.*, 1986; Bhatnagar *et al.*, 1986; Bohannon and Sonenshein, 1989; Brenchley *et al.*, 1975; Brown *et al.*, 1994; Fisher and Wray, 1989; Fuchs and Keister, 1980). According to rRNA sequence data, there appear to be two major archael subdivisions of GS: the Euryarchaeota, which includes halophiles, methanogens and some thermophiles, and the Crenarchaeota, which includes hyperthermophiles (Brown *et al.*, 1994). Within the first division, four distinct forms of GS have been identified thus far, namely GSI, GSII, GSIII and GlnT. Each of these is considered below, although particular emphasis is placed on GSI since this is the form found in *C. glutamicum*.

GSI: This is the most studied of all GS forms since its distribution is almost universal within the prokaryotes (Merrick and Edwards, 1995). It generally consists of 12 identical subunits which are encoded by the *glnA* gene. The *glnA* genes of many bacteria have been cloned, and the primary amino acid sequence of GSI is highly conserved across a wide range of genera (Woods and Reid, 1993). The GSI enzymes of prokaryotes can be further subdivided into the GSI-α and GSI-β families (Brown *et al.*,

1994). GSI-α is found in the low G+C Gram-positive bacteria, such as members of *Bacillus* (Deuel and Stadtman, 1970), *Clostridium* (Janssen *et al.*, 1988) and the Euryarchaeota (which include methanogens, halophiles, and some thermophiles). GSI-β, on the other hand, occurs in all other bacteria including the *Enterobacteriaceae* (Brown *et al.*, 1994), *Vibrio alginolyticus* (Bodasing *et al.*, 1985), *Thiobacillus ferrooxidans* (Barros *et al.*, 1986), *Streptomyces cattleya* (Streicher and Tyler, 1981), and *Streptomyces coelicolor* (Wray and Fischer, 1988). In addition to primary sequence differences, the subdivisions are generally based on two criteria: 1) the occurrence of a specific 25 amino acid insertion, which is present only in GSI-β, and 2) the possible presence of adenylylation control of the enzyme. Adenylylation occurs only in the GSI-β form, which is characterised by the occurrence of a short stretch of conserved amino acids. Regulation of GS activity by adenylylation is discussed in section 1.4.3.2.

GSII: This form of GS was originally identified in *Rhizobium* strains (Darrow and Knotts, 1977) and subsequently in *Agrobacterium* (Fuchs and Keister, 1980). This enzyme is encoded by the *glnII* gene, and differs markedly from GSI in that it is an octamer of identical subunits that are arranged in two discs (Carlson and Chem, 1986). In terms of its holoenzyme structure, temperature lability, and lack of adenylylation regulation, GSII is similar to the eukaryotic enzyme. The GSII subunits are polypeptides 329 to 373 amino acids in length, which is approximately 100 amino acids shorter than those of GSI (Woods and Reid, 1993) Despite the lack of an adenylylation site, there is some evidence that GSII in *Rhizobium leguminosarium* may be subject to some other form of posttranslational modification (Behrmann *et al.*, 1990). GSII is found in members of *Rhizobiaceae*, *Frankiaceae*, *Streptomyces* (Brown *et al.*, 1994), and *Mycobacterium* (Harth and Horwitz, 1997), which are characterised by possessing both GSI and GSII. Both GS enzymes from *Rhizobiaceae* and *Frankiaceae* are regulated in response to nitrogen, and *glnII* is expressed in response to nitrogen starvation (Darrow and Knotts, 1977; Edmands *et al.*, 1987).

GSIII: The GSIII enzyme was initially identified in the Gram-negative aerobe *Bacteroides fragilis* (Hill *et al.*, 1989), and homologs have subsequently been found in *Butyrivibrio fibrisolvens* (Woods and Reid, 1993) and *Synechocystis* strain PCC 6803

(Reyes and Florencio, 1994). This GS is a hexamer with a subunit molecular mass of 75 kDa. The amino acid sequence of GSIII differs from that of GSI and GSII, although several conserved regions near the active site have been identified. The *B. fibrisolvens* GS has a large subunit of 701 amino acids and shows 40% identity to that of *B. fragilis* (Woods and Reid, 1993). Like *glnA*, *glnN*, the gene encoding GSIII in *Synechocystis*, is highly expressed in nitrogen-starved cells (Reyes *et al.*, 1997).

For a detailed discussion of the evolutionary relationships of GSI, GSII and GSIII see Brown *et al.* (1994).

GlnT: In addition to GSI and GSII, *R. leguminosarium* harbours yet another form of the GS enzyme, which has variously been called GlnT (Merrick and Edwards, 1995). This enzyme has also been identified in *R. meliloti* and is encoded by the *glnT* gene. Like GSII, GlnT is an octamer, with a subunit molecular mass of 47 kDa. In contrast to GSII, however, GlnT is stable at elevated temperatures.

*1.4.3.2 Regulation of GSI activity*

Feedback inhibition: The activity of GS purified from *E. coli* grown in nitrogen-limited medium is inhibited by a number of compounds including serine, histidine, tryptophan, CTP, AMP, alanine, glycine, carbamyl-phosphate and glucosamine 6-phosphate (Reitzer, 1998). Alanine, glycine and serine belong to the class I inhibitors since they acquire their nitrogen from glutamate, which may obtain its nitrogen from glutamine (Stadtman, 1990). The synthesis of the other six inhibitors, on the other hand, absolutely requires glutamine, and these belong to class II. It appears that class I and II inhibitors affect GS activity by different mechanisms. It has been suggested that class I inhibitors bind to a single site, while each class II inhibitor binds to a single allosteric site (Ginsburg and Stadtman, 1973). Interestingly, the susceptibility to feedback inhibition is affected by the nitrogen concentration of the medium (Kingdon and Stadtman, 1967). GS obtained from nitrogen-starved cells is less sensitive to feedback inhibition than GS from cells grown in nitrogen-rich medium. This differential inhibition is the direct result of the covalent modification of the GS enzyme by adenylylation.

Feedback inhibition is widespread in enzymes that are involved in amino acid synthesis (Reitzer, 1998). For example, aspartokinase, an enzyme involved in the synthesis of aspartate family amino acids in *C. glutamicum*, is inhibited by L-lysine and L-threonine (Malumbres and Martin, 1996). Ertan (1992a) has shown that GS activity is indeed regulated by cellular pools of alanine, glycine and serine in *Corynebacterium callunae*. Presumably, similar regulation occurs in *C. glutamicum*. The cumulative inhibition by various combinations of these amino acids indicates that these modifiers each have their own binding site on the GS enzyme. Similar GS inhibitory patterns have been reported for *Chlorobium vibrioforme* (Khanna and Nicholas, 1983).

Adenylylation: This posttranslational modification is the covalent addition of an AMP moiety to a conserved tyrosine residue of each subunit of the GS enzyme (Woods and Reid, 1993). Once adenylylated, that specific subunit is inactive such that the enzyme can exist in a range of activity states within the cell (Stadtman, 1990). Adenylylation occurs in response to high nitrogen conditions, and progressive deadenylylation occurs as nitrogen becomes more limiting, thereby activating the enzyme. Both adenylylation and deadenylylation are catalyzed by the monomeric adenylyltransferase enzyme (ATase), which is around 130 kDa in size (Ebner *et al*, 1970.). In addition to ATase, the adenylylation cascade of *E. coli* involves the bifunctional uridylyltransferase (UTase)/uridylyl-removing enzyme (UR) and $P_{II}$ (Anderson and Stadtman, 1971). $P_{II}$ is encoded by the *glnB* gene and is present in a uridylylated ($P_{II}$-UMP) or a deuridylylated state ($P_{II}$)(Fig. 1.4).



The interconversion between $P_{II}$ and $P_{II}$-UMP is catalysed by UTase/UR which is regulated in response to nitrogen levels. $P_{II}$ is present when nitrogen is sufficient (high glutamine: α–ketoglutarate ratio) and stimulates the adenylylation reaction of ATase (Brown *et al.*, 1971). Conversely, when nitrogen is limiting (low glutamine:α–ketoglutarate ratio), $P_{II}$-UMP stimulates the deadenylylation activity of ATase which in turn inactivates GS. This regulatory system is revisited in a later section dealing with nitrogen control in *E. coli* (section 1.5)

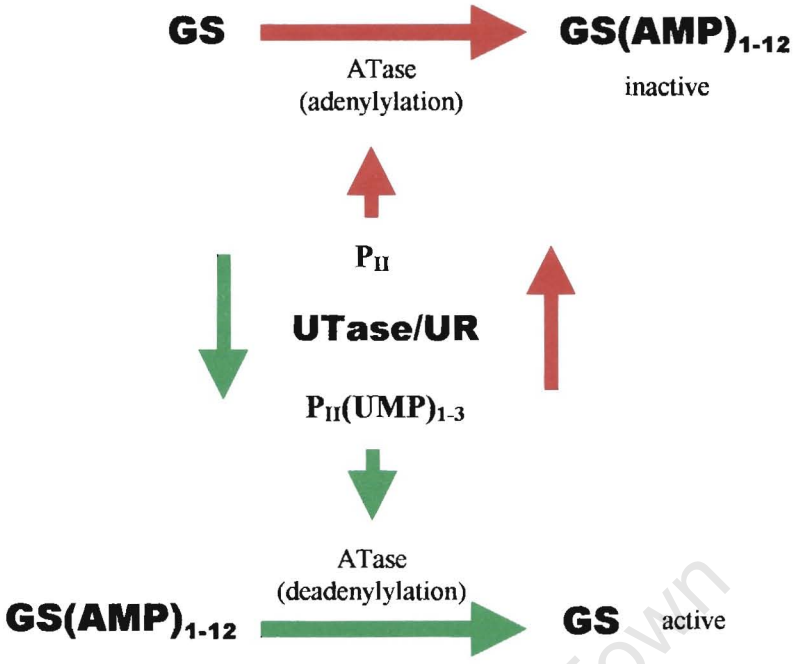



**Fig. 1.4** Cyclic adenylylation of GS. Red arrows depict events that occur in response to high nitrogen conditions, while green arrows depict events occurring during nitrogen starvation (adapted from Reitzer, 1998).

Despite the reversible nature of the adenylylation process, the deadenylylation reaction is not chemically the reverse of the adenylylation reaction (Ebner *et al.*, 1970; Ginsburg and Stadtman, 1973). The adenylylation reaction is as follows:

$$GS + nATP \rightarrow GS\text{-}AMP_n + nPP_i$$

The phosphate-dependent deadenylylation reaction, on the other hand, is as follows:

$$GS\text{-}AMP_n + nP_i \rightarrow GS + nADP$$

Apart from inactivating specific subunits, adenylylation also affects GS in other ways. Firstly, partial adenylylation makes the unadenylylated subunits more susceptible to feedback inhibition (Shapiro *et al.*, 1967). Secondly, adenylylation affects metal ion specificity and the pH activity profile of GS (Kindon and Stadman, 1967).

Transcriptional regulation: In addition to covalent modification, GSI activity is often regulated at the level of transcription, the classical case being that of enteric bacteria (Merrick and Edwards, 1995). In general, nitrogen-rich conditions repress *glnA* transcription in bacteria, while nitrogen starvation induces transcription (reviewed by Reitzer, 1998). The exact manner in which *glnA* transcription is regulated in response to nitrogen status differs among the various bacteria, and selected regulatory systems are discussed in sections 1.4 and 1.5.

### 1.4.4 Glutamate synthase

The glutamate synthase enzyme, previously known as glutamine amide-2-oxoglutarate amidotransferase (GOGAT), was initially discovered in *Klebsiella aerogenes* (Tempest *et al.*, 1970). Prior to this discovery, the only route of glutamate synthesis from ammonia was by the GDH pathway (Meers *et al.*, 1970). GOGAT activity has since then been discovered in a large variety of bacterial genera and higher organisms including algae, fungi, yeast, and higher plants (Vanoni and Curti, 1999). This enzyme is characterised as catalyzing the synthesis of two molecules of glutamate from one molecule of glutamine and α-ketoglutarate (Castaño *et al.*, 1992). This reaction places the GOGAT enzyme at an important intersection of nitrogen and carbon metabolism. As is the case in GS, various classes of bacterial GOGAT enzymes can be distinguished.

#### *1.4.4.1 Classes of GOGAT enzymes*

The first class of GOGAT enzymes is dependent on the reduced pyrimidine nucleotides NADH or NADPH. This enzyme consists of a large and small subunit, which are encoded by the *gltB* and *gltD* bacterial genes, respectively. Strangely, the corresponding genes have been designated *gltA* and *gltB* in *B. subtilis* (Bohannon *et al.*, 1985). The molecular weight of the small (β) subunit is around 55 kDa in all organisms, while the large (α) subunit shows more variation and ranges from 135 kDa to 175 kDa (Reitzer, 1996). The α and β subunits together form the active protomer which is approximately 200 kDA in size (Vanoni and Curti, 1999). Some forms of GOGAT display a quarternary structure of the type $(\alpha\beta)_4$ as in *E. coli* and *A. brasilense*, while others are of the αβ conformation and do not aggregate to form a high MW species.

Some studies suggest that purified GOGAT is an octamer, while others suggest it is a dimer that aggregates during purification (Miller and Stadtman, 1972; Trotta *et al.*, 1974). The protomeric structure of GOGAT also seems to depend on the organism in question. The purified bacterial enzyme contains one flavin adenine dinucleotide (FAD), one flavin mononucleotide (FMN), and three different iron-sulfur clusters (Vanoni and Curti, 1999). In *Klebsiella aerogenes*, the large subunit binds glutamine and the ironsulfide flavin (Mantsala and Zalkin, 1976a; Mantsala and Zalkin, 1976b). The rather large size of this glutamine-binding subunit probably stems from its participation in electron transfer, which is considered an unusual property for an amidotransferase. The large subunit displays glutaminase activity (Buchanan, 1973), however a variety of treatments that inactivate or damage this glutaminase activity have no effect on the ammonia-dependent activity (Geary and Meister, 1977; Mantsala and Zalkin, 1976a-c). In *E. coli*, the purified small subunit can catalyze the ammonia-dependent synthesis of glutamate (Mantsala and Zalkin, 1976a). In *Salmonella typhimurium*, however, a *gltB-gdh* mutant, which has only the large GOGAT subunit and no GDH, displays a phenotype characteristic of a GOGAT-deficient strain and requires glutamate for growth (Madonna *et al.*, 1985). It seems, therefore, that the GDH-like activity displayed by the small subunit is limited to *in vitro* conditions and is not physiologically significant.

The reaction catalyzed by the GOGAT enzyme occurs in two distinct steps. First, the enzyme-bound flavin is reduced by NAD(P)H, after which the reduced flavin reacts with α-ketoglutarate to generate oxidized flavin and two molecules of glutamate (Geary and Meister, 1977; Mantsala and Zalkin, 1976c). This reaction implies that both subunits are necessary for the catalytic activity, consistent with the genetic evidence mentioned above. Most bacteria posses an NADPH-dependent GOGAT, some an NADH-dependent form, while others still posses a GOGAT that has dual cofactor utilisation (Ertan, 1992). The GOGAT of *C. callunae*, for example, has such dual cofactor utilisation, but NADH-dependent activity is only observed under certain conditions and is substantially lower than NADPH-dependent activity.

GOGAT enzymes belonging to the second class are found in plants (Avila *et al.*, 1993; Knaff *et al.*, 1991) and photosynthetic cyanobacteria (Marques *et al.*, 1992) and require reduced ferredoxin as their electron donors. This particular form of GOGAT, which shows significant homology with the bacterial α subunit, occurs as a homodimer where the subunit size varies from 125 to 180 kDa.

The third class of GOGAT comprises the eukaryotic, pyridine-dependent form which is found in fungi, yeast and non-photosynthetic tissues (nodules) of plants (Gregerson *et al.*, 1993). This form of the enzyme is composed of a single polypeptide approximately 200 kDA in size and appears to derive from the fusion of the bacterial α and β subunits. In *Saccharomyces cerivisiae*, GOGAT exists as a homodimer comprising three 199kDA polypeptide monomers (Congoni *et al.*, 1995). In plants on the other hand, the enzyme is composed of four identical monomers of around 220 kDA (Anderson *et al.*, 1989).

*1.4.4.2 Regulation of GOGAT activity*

Regulation of GOGAT activity has been most extensively studied in *E. coli* (Garciarrubio *et al.*, 1983; Lozoya *et al.*, 1980). The two GOGAT structural genes, together with *gltF*, form the *gltBDF* operon, which is located at 69 min on the *E. coli* chromosome (Garciarrubio *et al.*, 1983). As is the case with GS, cellular GOGAT activity is often dependent on a number of factors, including nitrogen and carbon status (Reitzer *et al.*, 1998). In the *Enterobacteriaceae*, two primary regulatory phenomena have been observed. Firstly, GOGAT activity is high in ammonia-containing medium (Brenchley *et al.*, 1975). Secondly, glutamate or compounds that generate glutamate, repress *gltBDF* transcription in nitrogen-limited media (Brenchley *et al.*, 1975; Miller and Stadtman, 1972).

The product of the *gltF* gene was initially thought to be involved in the glutamate-dependent repression of the *gltBDF* operon (Castano *et al.*, 1992). However, the subsequent generation of a non-polar *gltF* insertion mutant revealed that this strain was not impaired in amino acid or methylammonium transport or utilization (Grassl *et al.*,

1999). Further investigation showed that GltF is in fact a periplasmic protein, and is not involved in the transcriptional control of *gltBDF* or any other nitrogen metabolic genes.

Expression of the *gltBDF* operon is not regulated by the global Ntr system (Reitzer and Magasanik, 1987). In contrast to other Ntr enzymes, GOGAT activity is greatly reduced by growth in glutamate, yet enhanced to the same extent by growth in either excess ammonia or a nitrogen-limiting source such as glutamine (Castaño *et al.*, 1988). Instead, expression of the *gltBDF* operon in *E. coli* is positively regulated by the leucine-responsive regulatory protein (Lrp)(Ernsting *et al.*, 1993).

Lrp positively regulates *glnA* and the *gltBDF* operon, as well as many other genes involved in nitrogen metabolism (Ernsting *et al.*, 1992). Pyridine nucleotide transhydrogenase expression is also positively regulated, in keeping with the demand for NADPH during amino acid biosynthesis and ammonia assimilation. However, Lrp also negatively regulates several operons involved in amino acid catabolism and nutrient transport. Lrp exerts its control by binding directly to specific sites in the promoter regions of the target genes. Leucine serves as a coregulator that modulates the activation or repression of these genes (Calvo and Matthews, 1994). It has been suggested that leucine affects the affinity of Lrp binding to its target DNA without apparently affecting the sequence specificity of binding (Ernsting *et al.*, 1993). The extent of this effect is a function of 1) the intrinsic affinity of the specific target sequence for Lrp, where leucine-insensitive genes, such as the *gltBDF* operon, exhibit a higher affinity for the regulator than leucine-sensitive genes, and 2) the intracellular concentration of the Lrp protein. The physiological significance of Lrp-mediated regulation of GOGAT activity is discussed in detail by Reitzer (1998).

In addition to nitrogen regulation, GOGAT activity in the *Enterobacteriaceae* is also affected by carbon availability, as well as the stringent response (Reitzer, 1998). Furthermore, it has been shown that carbon limitation moderately represses GOGAT synthesis, and, interestingly, a CRP-binding site overlaps the –35 RNA polymerase binding site of the *gltBDF* operon, suggesting CRP regulation (Oliver *et al.*, 1987).

### 1.4.5 Glutamate dehydrogenase

GDH purified from *E. coli* and *S. typhimurium* revealed that this enzyme is a hexamer of identical subunits. The encoding gene, *gdhA*, has been most extensively characterised in these two organisms and is monocistronic and contains a single promoter (Reitzer, 1998). GDH activity in *E. coli* is high in cells grown in glucose-ammonia minimal medium, and is low when glutamate or aspartate is present (Brenchley *et al.*, 1975; Halpern and Umbarger, 1976). In *S. typhimurium*, on the other hand, exogenous aspartate, but not glutamate, represses GDH activity (Brenchley *et al.*, 1975).

Carbon limitation in *E. coli* results in reduced GDH activity (Halpern and Umbarger, 1960). A CRP-binding site overlaps the –35 RNA polymerase binding site, which implies that the regulation involves steric blocking of RNA polymerase binding. This type of regulation may prevent removal of excessive α-ketoglutarate from the citric acid cycle during growth under carbon limitation.

During nitrogen limitation, GDH synthesis is strongly repressed in *K. aerogenes* (Bender *et al.*, 1976), moderately in *E. coli* (Riba *et al.*, 1988) and not at all in *S. typhimurium* (Brenchley *et al.*, 1975). The Nac (nitrogen assimilatory control) protein is the transcriptional regulator that is responsible for this control in *K. aerogenes*. Nac also activates expression of the Ntr genes including those involved in proline, histidine and urea utilization, while Nac itself is also synthesized as part of the Ntr response (Bender, 1991). The actual activity of the Nac protein, however, does not appear to be under any form of control. Nac may function to buffer the control of some Ntr genes from the highly sensitive regulators of the Ntr response, $P_{II}$ and UTase/UR. Thus, even when nitrogen-starved cells are removed to a nitrogen-rich environment, Nac-dependent regulation would persist until Nac is sufficiently diluted. Such insensitivity may be desired once a commitment has been made to the Ntr reponse (Schwacha and Bender, 1993).

Several researchers have investigated the importance of GDH in *C. glutamicum* and bacteria in general, since the GS/GOGAT pathway also generates glutamate. Many

bacteria use both the GS/GOGAT and GDH pathway for ammonia assimilation, while others have been found to use only one or the other (Ertan, 1992b). GDH-deficient strains of *E. coli, K. aerogenes* and *S. typhimurium* display no discernible growth defect in energy-rich medium (Brenchley and Magasanik, 1974), although *E. coli* mutants are at a competetive disadvantage in energy-limited conditions (Helling, 1994). GDH mutants also seem to have an enhanced sensitivity to glutamate analogs (Brenchley, 1973). In *C. glutamicum* ATCC 13032, the strain used in our studies, it seems that GDH is employed under high ammonia and phosphate conditions and when the cell is energy/carbon limited (Helling, 1994). Ertan (1992a) found that GDH activities in *C. callunae* were not significantly affected by the type or concentration of the nitrogen source. Interestingly, the GDH responses to nitrogen are both species- and strain-specific. In *C. glutamicum* ATCC 13058, for example, GDH activity significantly decreased with increasing ammonium concentrations. Similary, *gdh* transcription in *C. diptheriae* has been shown to increase in response to nitrogen starvation (Nolden *et al.*, 2002). In the case of *C. glutamicum* ATCC 13032, there seem to be conflicting reports as to the effect of nitrogen status on the activity of GDH. Extensive metabolic flux experiments performed by Tesch *et al.* (1999) demonstrate that GDH levels show only a weak dependency on nitrogen or carbon status in *C. glutamicum* ATCC 13032, while more recent unpublished experiments performed by Nolden (referred to in Nolden *et al*, 2002), suggest that *gdh* transcription is significantly enhanced in this strain under nitrogen starvation. Clearly, GDH regulation in *C. glutamicum* 13032 is not fully understood at present.



In addition to being synthesized by the action of GDH and GOGAT, glutamate production also occurs via two other pathways involving the enzymes glutaminase and transaminase (Schreier, 1993).

### 1.4.6 Glutaminase

The production of glutamate by the catabolism of glutamine is catalysed by the enzyme glutaminase, also known as L-glutamine aminohydrolase (Schreier, 1993). *E. coli* has two glutaminase activities. Glutaminase A is an inducible enzyme that responds to

cAMP, nitrogen compounds, and growth stage, while glutaminase B is a constitutive activity that is most likely the sum of all cellular glutaminases associated with amidotransferases (Pruisner, 1973). The control of glutaminase activity is important in ammonia assimilation, since significant levels of both activities are present when cells are ammonia-limited. GS activity is also high under these conditions, and it is not understood how *E. coli* regulates glutaminase activity to avoid futile cycling.

Glutaminase activity has also been detected in *B. subtilis* (Fisher and Sonenschein, 1984) and *Bacillus licheniformis* (Cook *et al.*, 1981). *B. licheniformis* has two distinct glutaminase activities which are active at different pHs. The activity at pH 7 is similar to the glutaminase B activity of *E. coli* and is essentially constitutive. The activity at pH 9, on the other hand, was found to be 10-fold higher in cells grown in glucose-glutamine medium than those grown in glucose-ammonia medium. The activity at pH 9 is therefore similar to the *E. coli* glutaminase A activity. Thus, it appears that the glutaminase A-like activity is used for glutamine catabolism and may be regulated by a carbon metabolite repression mechanism (Cook *et al.*, 1981).

### 1.4.7 Transaminases

Glutamate-α-ketoglutarate transaminase and glutamate-pyruvate transaminase activities have been detected in a number of *Bacillus* species (Charba and Nakata, 1977; Meers and Pedersen, 1972; Aronson, 1975; Kanamori *et al.*, 1987) throughout growth and sporulation (Charba and Nakata, 1977). These enzymes transfer an amino moeity to α-ketoglutarate and pyruvate, respectively, generating glutamate in the process. This reversible transfer of an amino group between amino acids and the alpha-keto acids, and the fact that these activities are constitutive, suggest that they serve to maintain an equilibrium between the amino acid and α-keto acid pools (Schreier, 1993). In enteric bacteria, three classes of transaminases have been identified. These transaminases result in the utilisation of keto acids to form amino acids (Reitzer and Magasanik, 1987).

The overall effect of glutaminase and transaminase activity on the cellular glutamate pool is difficult to assess, especially since their regulation remains elusive. However,

the moderate expression of GOGAT in *B. subtilis* and *B. licheniformis* in medium containing glutamine suggests that the contribution of glutaminase may not be significant (Schreier, 1993).

## 1.5 Nitrogen Control in *E. coli*

Nitrogen regulation in *E. coli* has been extensively studied and will only be discussed here briefly for comparative purposes. For thorough reviews see Merrick and Edwards (1995) and Reitzer (1998).

Ammonia is considered the preferred nitrogen source for the *Enterobacteriaceae* and results in the repression of proteins that catabolise other nitrogen sources (Gutnick *et al.*, 1969; Tyler, 1978). In *E. coli*, the *glnA* gene forms part of the *glnALG* operon also known as the *glnA-ntrBC* operon which is regulated by the Ntr (nitrogen-regulated) system (Hirschman *et al.*, 1985). The Ntr response regulates the expression of a number of proteins involved in nitrogen metabolism including those involved in the transport and catabolism of arginine, tryptophane, asparagine, proline, and glutamine, the degradation of urea by urease, and the assimilation and utilization of nitrate and nitrite by reductases (Lin and Stewart, 1998). Furthermore, the Ntr system also activates genes that encode secondary transcriptional activators. The activity of the Ntr system is controlled by a regulatory cascade (Fig. 1.5) that is responsive to intracellular nitrogen concentrations, where conditions of nitrogen limitation induce the Ntr response.

The Ntr system consists of a sensor protein ($NR_{II}$ or NtrB), a response regulator ($NR_I$ or NtrC), and RNA polymerase complexed to $\sigma^{54}$ (Ninfa and Magasanik, 1986). $NR_{II}$ monitors nitrogen status and is a bifunctional protein that can either transfer or remove phosphate from $NR_I$. Phosphorylated $NR_I$, on the other hand, is a transcriptional activator of $\sigma^{54}$-dependent promoters including *glnA* and other genes involved in nitrogen regulation and metabolism. The *glnA-ntrBC* operon contains three promoters (Ap1, Ap2 and *ntr*Bp, Fig. 1.5), which are used under different nitrogen conditions.

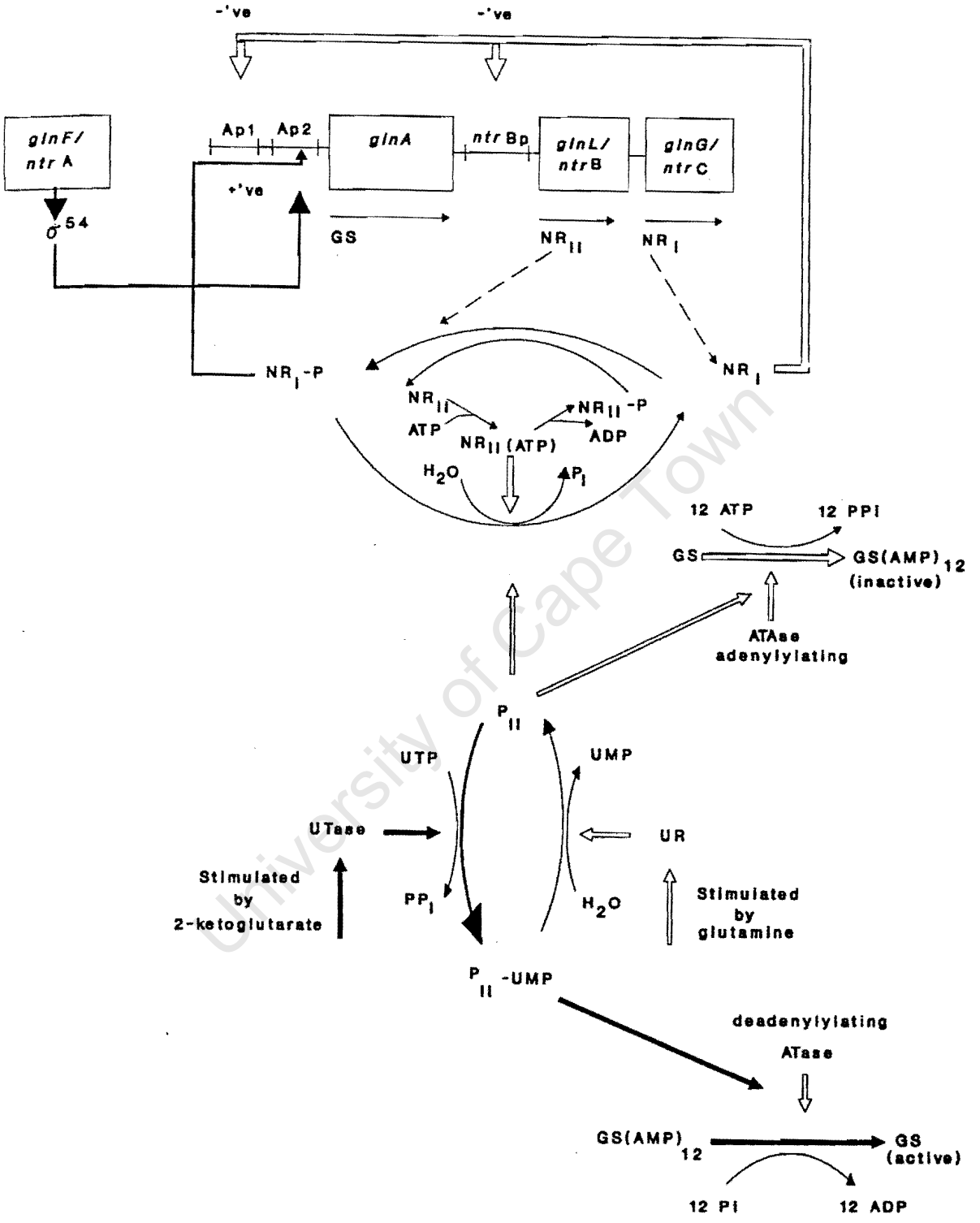





**Fig. 1.5** Regulation of the Enterobacteriaceae *glnA-ntrBC* operon. Open arrows: high glutamine to α-ketoglutarate ratio (high nitrogen); solid arrows: high α-ketoglutarate to glutamine ratio (low nitrogen). See text for explanation. Taken from Woods and Reid (1993).

The Ap1 and *ntrB*p promoters have consensus $\sigma^{70}$ sequences and are active at a basal level under nitrogen sufficiency, resulting in the production of low levels of GS, $NR_I$ and $NR_{II}$. Promoter Ap1 has a cyclic AMP (cAMP) receptor protein (CRP) binding site located upstream, and transcription from this promoter requires activation by the cAMP-CRP complex (Reitzer and Magasanik, 1985). Promoter Ap2, on the other hand, is a $\sigma^{54}$ promoter whose activity requires an enhancing protein, in this case $NR_I$ (Merrick, 1993).

Nitrogen limiting conditions (low α-ketoglutarate:glutamine ratio) result in phosphorylation of $NR_I$ which then binds to the Ap1 and *ntrB*p promoters and prevents transcription of *glnA* and *ntrBC* (Pahel *et al.*, 1982). The link between regulation of GS activity by adenylylation and transcription of *glnA* involves a pivotal protein, $P_{II}$, which is encoded by *glnB*. This protein is present in two states, uridylylated ($P_{II}$-UMP) or deuridylylated ($P_{II}$). The $P_{II}$ protein occurs as a tetramer and each subunit can be uridylylated at a specific tyrosyl residue (Rhee *et al.*, 1985). The addition or removal of a UMP group to $P_{II}$ is catalysed by the uridylyltransferase activity of the uridylyltransferase/uridylyl-removing (UTase/UR) enzyme, where low nitrogen stimulates UMP addition to $P_{II}$. $P_{II}$–UMP in turn stimulates the deadenylylating activity of the adenylyltransferase (ATase) that removes AMP groups from the GS enzyme and activates it. Conversely, high nitrogen results in the formation of $P_{II}$ that stimulates the adenylylation activity of the ATase as well as the removal of phosphate from $NR_I$ which then acts as a repressor of transcription of the operon.



As mentioned previously, it has been shown that GOGAT synthesis in *E. coli* is not directly regulated by the Ntr system, i.e. unlike other Ntr enzymes, GOGAT synthesis is strongly repressed by growth in glutamate but is activated by growth in excess ammonia or in a limiting nitrogen source such as glutamine (Castaño, 1988). Nonetheless, there seems to be a link between the Ntr network and the *gltBDF* operon.

GDH activity in *E. coli* is high in cells grown in glucose-ammonia minimal medium and is repressed by exogenous aspartate and glutamate (Reitzer, 1988). Nitrogen limitation, on the other hand, moderately represses GDH synthesis in *E. coli* (Riba *et al.*, 1988).

Unlike GS, GDH activity in this organism is not subject to regulation by the Ntr response.

## 1.6 Nitrogen Control in Gram-positive Bacteria

There is little evidence of a classical Ntr system in Gram-positive bacteria, although elements of an alternative global nitrogen regulatory system are present in *B. subtilis* (see section 1.6.1) and *Corynebacterium* (see section 1.7). The nitrogen regulatory mechanisms of selected Gram-positive bacteria are considered below, including those of *B. subtilis*, *Clostridium acetobutylicum*, *Mycobacterium tuberculosis* and *Streptomyces coelicolor*.

### 1.6.1 *Bacillus subtilis*

The review by Fisher (1999) was consulted extensively for this section.

As far as nitrogen metabolism is concerned, *B. subtilis* is probably the most well-characterized of all Gram-positive bacteria (Fisher, 1999). According to the enzymes employed in nitrogen assimilation, the genus *Bacillus* can be divided into three distinct groups. In 1993, Schreier classified *B. subtilis* as a member of the first group which employs both GS and GOGAT for nitrogen assimilation. Members of the second group utilize GS, GOGAT as well as GDH, and those of the third group use only the GDH, a characteristic of some nitrogen-fixing members of the genus. However, this may not be a true distinction, since GDH-like genes were later discovered in *B. subtilis* (Belitsky and Sonenshein, 1998).

The sequencing of the complete genome of *B. subtilis* has greatly aided the identification of novel genes and the characterisation of metabolic pathways. Although the genes for nitrogen metabolism are regulated by the availability of rapidly metabolizable nitrogen sources in *B. subtilis* (Pan and Coote, 1979), no system analogous to the enteric two-component Ntr control exists in this organism (Fisher, 1999). Unlike enteric bacteria, the expression of several amino acid catabolic enzymes

is not subject to nitrogen regulation; although these enzymes are substrate-inducible, the presence of a good nitrogen source does not repress their expression.

The GS enzyme of *B. subtilis* differs markedly from the enteric enzyme in primary sequence, susceptibility to carboxypeptidase A, and immunochemical properties. Expression levels of GS in *B. subtilis*, as in so many other bacteria, are related to the presence of readily available nitrogen sources (Deuel and Stadtman, 1970). The *glnA* gene of this organism lies in a dicistronic operon in which it is preceded by *glnR* whose gene product is required for the negative regulation of the operon (Brown and Sonenshein, 1996). The GlnR protein binds with high affinity to a DNA region that overlaps with the promoter of the *glnRA* operon. This binding to the promoter region of the operon prevents transcription of *glnRA* under high nitrogen conditions. GlnR alone, however, is not sufficient to regulate the operon in response to nitrogen status. Genetic evidence suggests that the GS enzyme is also required to effect this regulation and that of other nitrogen metabolism operons. In fact, Sonenshein and Brown (1996) have shown that GS has a stimulatory effect (two- to four-fold) on the binding of the *B. subtilis* GlnR protein to the *glnRA* promoter region. The mechanism of this stimulation, however, remains unclear. Furthermore, GS is also thought to be involved in sporulation, since certain mutations that result in constitutive GS expression also affect sporulation initiation (Schreier, 1993).

In the presence of glutamine, a preferred nitrogen source, GS levels are lowest. In contrast, moderate to high GS levels are observed when *B. subtilis* is grown in nitrogen limiting conditions or with non-preferred nitrogen sources. Although certain residues that occur around the *E. coli* GS adenylylation site also occur in the *B. subtilis* protein, regulation of the *Bacillus* GS via adenylylation has been ruled out by both *in vitro* and *in vivo* studies (Schreier *et al.*, 1985). The activity of the *B. subtilis* GS also appears to be regulated by feedback inhibition, although the enzyme is not as sensitive as the enteric one. The effects of various GS inhibitors are dependent on the divalent cation used for enzyme activation (Deuel *et al.*, 1971), and it has been suggested that the levels of $Mn^{2+}$ and $Mg^{2+}$ during growth and sporulation are relevant to the regulation of several key metabolic enzymes (Charney *et al.*, 1951).

In the case of GOGAT, enzyme levels respond both to the nature as well as the availability of the nitrogen source. The presence of glutamate or amino acids that are directly degraded to glutamate, such as histidine and arginine, result in reduced GOGAT levels (Pan and Coote, 1979). Interestingly, glutamine results in intermediate enzyme levels. Unlike GS, GOGAT activity is highest in the presence of ammonia. The *gltAB* genes, encoding the large and small subunit of GOGAT, are under the control of GltC which is a member of the LysR family of regulatory proteins (Bohannon and Sonenshein, 1989). The GltC protein is encoded by *gltC* which occurs upstream of and in the opposite orientation as *gltAB*. In addition to repressing it's own synthesis, GltC also activates transcription of *gltAB* under glutamate-limited conditions. The GOGAT of *Bacillus* spp. has a nearly absolute requirement for NADPH and is relatively insensitive to feedback inhibition (Schreier, 1993).

In addition to GlnR and GltC, two other regulatory proteins, CodY and TnrA, have been identified that control the expression of gene products involved in nitrogen metabolism in response to nutrient availability in *B. subtilis* (Fisher, 1999). When nitrogen is limiting, TnrA protein activates the transcription of a number of nitrogen genes, including urease, ammonium permease, and the nitrate assimilatory enzymes (Fisher, 1999). TnrA also functions as a negative regulator, repressing the expression of GS and GOGAT (encoded by *gltAB*) during nitrogen limited growth. In addition, TnrA positively regulates its own synthesis as well as that of some proteins not directly involved in nitrogen metabolism. TnrA is a homolog of GlnR, and both proteins belong to the MerR family of DNA-binding proteins. Their proposed N-terminal DNA-binding domains are nearly identical, and both proteins bind similar DNA sequences. Although the nitrogen signal regulating the activity of GlnR and TnrA is not known, the GS protein is required for the transduction of this signal to GlnR and TnrA. The other regulatory protein, CodY, negatively regulates several genes involved in nitrogen metabolism, including the histidine degradative operon (*hut*), the dipeptide transport operon (*dpp*), and the isoleucine/valine degradative operon (*bkd*) (Slack *et al.*, 1995).

Although the existence of GDH activity in *B. subtilis* had been a matter of controversy for some time, complete sequencing of the genome of this bacterium identified two

genes very similar to the GDH-encoding genes of other organisms (Belitsky and Sonenshein, 1998). Mutations in these two genes, *rocG* and *gudB*, revealed that the RocG is the major catabolic GDH, while GudB is an intrinsically inactive GDH. Expression of the *rocG* gene was induced in media containing arginine or ornithine or, to a lesser degree, proline, and was repressed by glucose. A *B. subtilis rocG* null mutant was impaired in media where arginine, ornithine or proline were the sole nitrogen or carbon sources. The *gudB* gene, on the other hand, was found to be expressed under all growth conditions tested, although the gene product was inactive. Interestingly, spontaneous mutations in *gudB* that removed a 9bp direct repeat within the wt *gudB* sequence activated the GudB protein and allowed more efficient utilization of amino acids of the glutamate family. Both GDH enzymes, when active, were found to utilise NAD(H) rather than NADP(H). It has been shown that cellular GDH activity modulates GltC activity which could in turn be related to the rate of α-ketoglutarate production (Belitsky, 1998). In *B. subtilis*, GDH levels are influenced by the presence of glutamate where enzyme levels are highest in rich medium with glutamate as the sole carbon and nitrogen source (Kane and Deshpande, 1979).

### 1.6.2 *Clostridium acetobutylicum*

Regulation of GS in *C. acetobutylicum* is markedly different from that in any other of the Gram-positive bacteria discussed here in that it involves antisense RNA control (Janssen *et al.*, 1990). The *glnA* gene of *C. acetobutylicum* can be expressed from either of two promoters (p1 and p2, Fig. 1.6), while a third promoter (p3) is located downstream of *glnA*. Four regions of dyad symmetry are present, three of which lie upstream of *glnA*, while the fourth overlaps with p2 (Janssen *et al.*, 1988). Transcription from p3 produces a short transcript complementary to a 43 bp region located at the start of the *glnA* mRNA (Woods and Reid, 1993). This antisense RNA (AS-RNA) binds to its complementary region thereby blocking the ribosome binding site and the *glnA* initiation codon. The result of this AS-RNA binding is prevention of translation of the *glnA* transcript and thus GS synthesis. All three promoters respond to nitrogen levels. However, the difference in activity of each promoter directs the production of GS such that under nitrogen-limiting conditions the level of *glnA* mRNA exceeds that of the AS-

RNA by around 5-fold so that GS protein is synthesized (Woods and Reid, 1993). Under nitrogen sufficient conditions, however, the situation is reversed, where 1.6-fold more AS-RNA is produced than *glnA* transcript and GS synthesis is thus prevented (Fierro-Monti *et al.*, 1992).

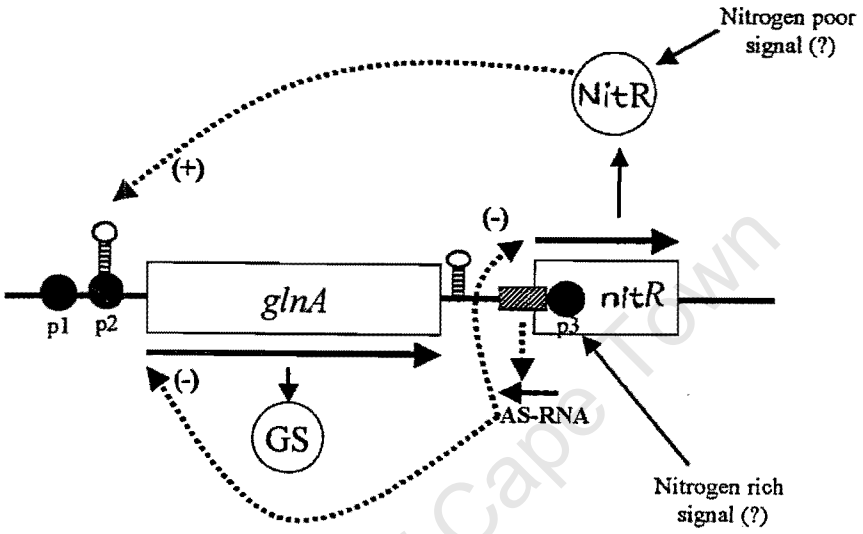



**Fig. 1.6** Regulation of the *glnA* region of *C. acetobutylicum* P262. Under nitrogen-limiting conditions, an unknown signal activates the putative transcriptional activator NitR to bind to the region of dyad symmetry overlapping promoter p2, thereby activating transcription of *glnA* from p1. When nitrogen is sufficient, transcription from promoter p3 is enhanced which directs the synthesis of the antisense mRNA. This transcript binds to the complemetary sequences present in the 5' regions of both the *glnA* and *nitR* mRNA transcripts, thus preventing their translation (taken from Stutz, 2000).



All three promoters conform to typical Gram-positive extended –10 and –35 promoter consensus sequences. However, since they are differently regulated, additional regulatory factors must be involved. Sequence analysis of the region directly downstream of the *C. acetobutylicum glnA* gene revealed the existence of an ORF of 566 nucleotides that is in the same orientation as *glnA* (Wood and Reid, 1995). The putative protein encoded by this ORF shows significant homology to the conserved domains of response regulators from two-component regulatory systems (Stutz, 2000). Under nitrogen limiting conditions, *glnA* and the putative regulator, designated NitR

(previously called glnR) are cotranscribed. Although the function of *nitR* has yet to be confirmed, a regulatory model for *glnA* regulation by NitR has been proposed.

It is suggested that under nitrogen limitation, a signal transduction mechanism induces expression of *nitR* whose gene product, NitR, acts as a transcriptional antiterminator that interacts with the stem-loop structure in p2 and induces *glnA* transcription. Under nitrogen sufficient conditions, p3 is activated resulting in the production of AS-RNA, which in turn binds to the 5' regions of the *glnA* and *nitR* mRNAs and reduces the synthesis of both GS and NitR. Therefore, GS activity in *C. acetobutylicum* is regulated at the level of transcription and translation. No evidence supporting posttranslational modification of GS has been reported in this organism (Schreier, 1993).

Recent studies by Stutz (2000) have shown that the genes encoding GOGAT in *C. acetobutylicum*, *gltA* and *gltB*, are located directly downstream of the *nitR* gene. Northern analysis revealed that that *gltA* and *gltB* are cotranscribed. GOGAT activities are regulated by nitrogen source in a similar way to GS, where activities were highest under nitrogen limiting conditions. GOGAT activities were specific for the cofactor NADH and sensitive to oxygen. No assimilatory GDH activity has been detected in *C. acetobutylicum*.



### 1.6.3 *Mycobacterium tuberculosis*

Nitrogen assimilation in *M. tuberculosis* has been the focus of several studies since it is closely linked to virulence. Moreover, the GSI enzyme has been identified as one of several potentially important pathogenic determinants of *M. tuberculosis* (Harth and Horwitz, 1997). This enzyme appears to influence the ammonia concentration within the phagosome containing the bacterium in host cells, and GS may be directly involved in the synthesis of poly-L-glutamic acid/glutamine found in abundance in the cell wall of pathogenic mycobacteria (Harth and Horwitz, 1994).

*M. tuberculosis* harbours four GS homologs (Parish and Stoker, 2000). The genes encoding two of these, *glnA1* and *glnA2*, are located in the same region of the

chromosome, and encode GSI and a putative GSII, respectively. In fact, the two *glnA* genes flank the gene encoding the adenylyltranferase enzyme, *glnE* (Fig. 1.7). The *glnA1* gene, however, is transcribed in the opposite direction as *glnA2* and *glnE*.

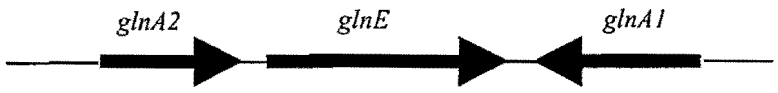



**Fig. 1.7** Genetic organization of the *glnA* cluster in *M. tuberculosis*.

The genes encoding the other two putative GS enzymes, *glnA3* and *glnA4*, are found elsewhere on the chromosome. These genes probably encode GSI and GSII enzymes, respectively, although their motifs are less conserved.

GSI activity in *M. tuberculosis* is profoundly affected by ammonia concentration, where a 10-fold increase in ammonium sulfate concentration results in an almost 10-fold reduction in GS activity. Regulation of the *M. tuberculosis* GS appears to be similar to that of *E. coli*, and involves posttranslational modification by adenylylation (Reitzer, 1998). In fact, the *glnE* gene was found to be essential in *M. tuberculosis*, since disruption mutants of this chromosomal gene could not be isolated unless another functional copy of *glnE* was provided (Parish and Stoker, 2000). The reason for this is not clear, but it has been suggested that constitutive GSI activity, a consequence of *glnE* disruption, results in the depletion of the intracellular pool of glutamate.

GS activity and production is inversely correlated to ammonia concentration, and *glnA* is transcribed from two promoters as a ~1.55 kb and a ~1.65 kb transcript. The nitrogen concentration affects transcription of *glnA* both quantitatively and qualitatively (Harth and Horwitz, 1997). Under low nitrogen conditions, the shorter transcript is synthesized in abundance while the longer one is absent. Under high nitrogen conditions, the longer transcript is produced in modest amounts while the shorter one is absent. The two transcripts appear to be initiated by two different sigma factors. High nitrogen conditions also result in adenylylation of GS by GlnE, such that GS activity is reduced. Interestingly, *M. tuberculosis* excretes around 33% of its GS enzyme, while

nonpathogenic mycobacteria do not display such a phenomenon (Harth and Horwitz, 1994).

### 1.6.4. *Streptomyces coelicolor*

Nitrogen regulation in *S. coelicolor* has particular relevance in the context of the research described here, since the GSI of *C. glutamicum* has 66% amino acid identity with that of *S. coelicolor* (Jakoby *et al.*, 1997). This homology suggests further similarities in nitrogen metabolism between these two organisms.

As mentioned in the previous section, a number of *Streptomyces* species have been shown to synthesize both GSI and GSII, and *glnII* genes have been cloned from a *S. hygroscopicus, S. viridochromogenes,* and *S. coelicolor* (Merrick and Edwards, 1995). In *S. coelicolor*, GSI activity is regulated at two levels. Firstly, transcription of *glnA* is regulated in response to nitrogen availability. The same promoter is employed during vegetative growth, stationary phase, and sporulation, and the gene is transcribed as a monocistronic mRNA. Secondly, the GSI enzyme is regulated by adenylylation, where high ammonium concentrations result in adenylylation of the enzyme and thus decreased activity. The adenylyltransferase gene (*glnE*) has been isolated from *S. coelicolor*, and GSI activity of *glnE* mutants was not downregulated when the cells were shocked with ammonium (Fink *et al.*, 1999). Since the GSII enzyme does not contain a conserved tyrosine residue, the site for adenylylation, it is assumed that this enzyme is not post-translationally modified in streptomycetes and that expression of *glnII* is constitutive (Hilleman *et al.*, 1993). In initial hybridization experiments to locate *glnE* on the *S. coelicolor* chromosome, the *glnE* fragment and the *S. coelicolor glnA* and *glnII* as well as a *glnB* fragment were used as probes (Fink *et al*, 1999). Southern blot analysis indicated that all of these genes are physically linked. This situation differs from that in *E. coli*, where *glnE* is physically separate from *glnA*, but is similar to *M. tuberculosis* where the *glnA*, *glnII* and *glnE* are adjacent. In *M. leprae*, *glnE* and *glnB* are colocalised, where homologs of these two genes were found downstream of *glnII* on the same cosmid.

In 1991, Wray's group identified six *S. coelicolor* mutants that required glutamine for growth at the wild-type rate on all nitrogen sources. Further investigation led to the isolation of the *glnR* gene. The product of this gene, a 29 kDa protein, was found to positively regulate *glnA* transcription, and has significant homology to other response regulators (Wray and Fisher, 1993). This is similar to the model of *glnA* regulation in *C. acetobutylicum* as proposed by Woods and Reid (1995). Interestingly, protein comparisons suggest that the GS enzyme of *S. coelicolor* is more similar to that of Gram-negative bacteria than of other Gram-positive bacteria such as *B. subtilis* and *C. acetobutylicum* (Wray and Fisher, 1988). Also, the occurrence of a *glnB* homolog in *S. coelicolor* suggests further similarity to the Gram-negative nitrogen control system.

In terms of GOGAT and GDH, both these enzymes are involved in the synthesis of glutamate in *S. coelicolor*. GOGAT activity is repressed in extracts of cells grown with good sources of glutamate, and the highest GDH activities were seen when cells were grown in high ammonium (Fisher, 1989). GOGAT activity is NADH- but not NADPH-dependent in this bacterium. Genetic studies have shown that GDH is used for assimilation when nitrogen is in excess, while the GS/GOGAT pathway is employed when nitrogen is limiting. This phenomenon is species-specific, however, and *S. clavuligens*, for example, assimilates ammonia exclusively via the GS/GOGAT pathway. No GDH activity is detectable under any physiological condition (Brana *et al.*, 1986).



## 1.7 Nitrogen Control in *C. glutamicum*

While this topic is the subject of the research described here, this section briefly deals with what is currently known about nitrogen regulation in *C. glutamicum*. Other aspects of this regulation are discussed in context in subsequent chapters.

Until recently, no proteins involved in the regulation of *glnA* and *gltBD* had been identified in *C. glutamicum*. A landmark discovery in *C. glutamicum* nitrogen control was made by Jakoby and coworkers when they identified the AmtR protein (Jakoby *et al.*, 2000). Previous studies by Siewe *et al.* (1996) had identified the *amt* gene whose

product was involved in (methyl)ammonium uptake in *C. glutamicum*. However, this uptake system was only active during nitrogen deprivation. It was therefore assumed that *amt* transcription was regulated in response to nitrogen status. Jakoby and colleagues (2000) therefore proceeded to establish a screening assay and successfully isolated the gene encoding the *amt* repressor, AmtR. This protein had significant similarity to members of the TetR/ArcR family of transcriptional regulators especially in the helix-turn-helix domain. Northern analysis of the *amtR* transcript revealed that this gene is monocistronic and transcribed at a low, constitutive level. The function of the repressor protein was confirmed by studying *amtR* disruption mutants; these strains displayed constitutive *amt* transcription.

Gel retardation experiments showed that AmtR binds to the upstream region of *amt*. Two palindromic DNA sequences (*amt*$_1$ and *amt*$_2$; Fig. 1.8) were located upstream of the *amt* coding region. AmtR was found to bind to both sequences when cells were grown in nitrogen excess. Interestingly, the *amt* gene has two transcriptional start sites ($P_1$ and $P_2$, Fig. 1.8). The reason for this is not known.



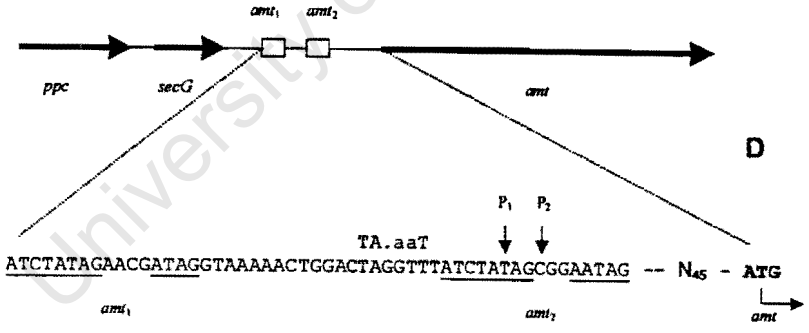



**Fig. 1.8** Genetic organization of the *amt* upstream region. The AmtR binding motifs, *amt*$_1$ and *amt*$_2$, and the ATG start codon of *amt* are shown. $P_1$ and $P_2$ indicate transcriptional start sites. A putative –10 consensus region is indicated based on promoter analysis performed by Pátek *et al.* (1996). No –35 consensus region could be identified. Taken from Jakoby *et al.* (2000).

In addition to regulating *amt* expression, AmtR was also shown to control the *amtB-glnK-glnD* operon. The genes of this operon encode an ammonium uptake gene (a homologue of *amt*), the signal transducing protein $P_{II}$, and the uridylyltransferase/uridylyl-removing enzyme, respectively. By regulating *glnK* and *glnD* expression, AmtR controls the uridylylation/deuridylylation cascade and thus the

adenylylation and resultant activity of GS, as is the case in *E. coli*. Only one copy of the AmtR binding motif was found upstream of *amtB*. The region that AmtR binds to during nitrogen excess consists of the DNA sequence ATCTATAG$N_{1-4}$ATAG. Upon nitrogen starvation, AmtR is released from its target sequence allowing transcription, although the signal for AmtR release is unclear (Jakoy *et al.*, 2000). Preliminary investigations have excluded low molecular weight metabolites such as glutamine, glutamate or α-ketoglutarate, and are more indicative of a protein-protein interaction mechanism. Jakoby *et al.* (2000) go on to suggest that the signal transducing protein $P_{II}$ may be a candidate for such interactions.

Based on sequence analysis, no AmtR binding motifs were found upstream of the *C. glutamicum gdh*, *glnA* and *amtR*. This is not surprising in the case of *gdh* or *amtR*, since transcription of the former is not significantly affected by nitrogen or carbon status (Tesch *et al.*, 1999), while expression of the latter is constitutive (Jakoby *et al.*, 2000). In the case of *glnA*, however, Nolden and colleagues (2001) recently used gel retardation experiments to show that AmtR was able to bind the motif ATCTAT, and thereby also represses *glnA* transcription in *C. glutamicum*. As a result, deletion of the *amtR* gene causes constitutive *glnA* expression. Moreover, it has now also been shown that AmtR regulates the expression of the *C. glutamicum* GOGAT genes *gltBD* (Beckers *et al*, 2001). In summary then, the AmtR protein controls the transcription of most nitrogen-regulated genes including *amt*, *amtB*, *glnK*, *glnD*, *glnA*, and *gltBD*, thereby establishing the role of AmtR as a global nitrogen repressor protein in *C. glutamicum* (Beckers *et al.*, 2001). A model for AmtR regulation has been proposed in Chapter 5.

Recent research by Nolden and colleagues has shown that a nitrogen regulatory system analogous to the one described above is present in *C. diptheriae* (Nolden *et al.*, 2002). Analysis of the genome database of *C. diptheriae* lead to the identification of homologs of various nitrogen genes including *amtB*, *glnE*, *glnK*, and *glnD*, as well as the gene encoding the global nitrogen regulator, *amtR*. Three of these genes were shown to form part of the *amtB-glnK-glnD* operon whose expression was increased when nitrogen was limiting. Northern hybridization studies revealed that expression of *amtR* in *C.*

*diptheriae* is constitutive, while *gdh* and *glnA* transcription is elevated in response to nitrogen starvation. In essence, this nitrogen regulatory cascade appears very similar to the one previously characterized in *C. glutamicum*. Surprisingly, the *gltBD* operon could not be unequivocally identified in *C. diptheriae* based on sequence analysis.

In contrast to other bacterial systems, *glnA* in *C. glutamicum* is transcribed as monocistronic mRNA, and this gene is not flanked by any genes involved in its regulation or nitrogen metabolism (Schulz, 1996). Whether this is also the case with the GOGAT-encoding genes is investigated within the scope of this research

## 1.8 Research Aims

The overall aim of this research was to further elucidate nitrogen metabolism in *C. glutamicum*. Particular emphasis was placed on studying the control of *glnA* and *gltBD* expression and regulation of the enzymes they encode, GS and GOGAT, respectively. Sequences adjacent to the aforementioned genes were investigated to determine whether they were involved in their regulation. Various molecular techniques were also employed in an attempt to identify novel genes involved in nitrogen regulation in *C. glutamicum*.

# Chapter 2

## The search for *glnII, glnR, glnE* and other nitrogen regulatory genes in *C. glutamicum*


### Abstract

Various strategies were employed to search for genes involved in nitrogen metabolism or regulation in *C. glutamicum*. Southern hybridizations were used to identify possible homologs of GlnR and GSII in *C. glutamicum* using the *Streptomyces glnR* and *glnII* genes as heterologous probes. However, even at a low temperature of 40°C no hybridization was detected. Degenerate primers were designed based on conserved regions within known GlnE proteins. However, none of the amplification products, obtained under various PCR conditions, yielded the desired *glnE* sequence. Two methods were used to search for novel genes involved in nitrogen metabolism. First, transposon mutagenesis was attempted in *C. glutamicum* using pTNC15, however with limited success. Similarly, screening an existing mini-library of *C. glutamicum* transposon mutants which were generated using Tn*5531* did not result in the isolation of desired mutants. Secondly, a *C. glutamicum* promoter probe library was created in the promoterless *cat* vector pEKplCm. Screening on 2mM and 100mM $NH_4Cl$ minimal medium plates, chloramphenicol MIC studies and chloramphenicol acetyltransferase (CAT) assays resulted in the identification of several nitrogen-responsive promoters. Some of these promoters belonged to genes encoding proteins involved in nitrogen metabolism, including GS, methionine aminopeptidase, and gamma cystathione synthase. Two promoters belonging to genes of unknown function that seemed to respond to nitrogen were further investigated under various nitrogen conditions. This second round of CAT assays provided no additional clues to the functions of the two sequences, although they were slightly responsive to nitrogen status.

## 2.1 Introduction

Our initial studies on nitrogen metabolism in *C. glutamicum* focused on characterization of the *glnA* gene, since this was the only cloned nitrogen assimilatory gene available from this organism at the time. This *glnA* gene had originally been isolated from *C. glutamicum* 13032 by complementation of the *E. coli* GS auxotroph YMC11 (Oldfield and Kenyon, 1995). Subsequent sequence analysis indicated that the GSI protein encoded by this gene had high amino acid identity to the GSI enzymes of *M. tuberculosis* (69% identity) and *S. coelicolor* (66% identity)(Schulz, 1996). The GS enzymes of both of these organisms have been studied extensively (see sections 1.6.3 and 1.6.4), and both are regulated at the transcriptional level as well as at the posttranslational level via adenylylation (Reitzer, 1998). The similarities between these enzymes suggest that other nitrogen metabolic and regulatory genes that have been identified in *M. tuberculosis* and *S. coelicolor* may also occur in *C. glutamicum*. Examples of such genes include *glnII*, *glnR* and *glnE*.

As discussed previously, the occurrence of two or even more forms of the GS enzyme in the same organism is not uncommon. *R. leguminosarium*, for example, harbours GSI, GSII and GlnT (Merrick and Edwards, 1995), while *M. tuberculosis* has four homologs of the enzyme (Parish and Stoker, 2000). Although the function of each GS is not fully understood, it is reasonable to assume that either they function under different physiological conditions, or they are evolutionary remnants.

The *glnR* gene was isolated from *S. coelicolor* over a decade ago (Wray *et al.*, 1991). During the course of this isolation, the researchers generated random mutants and studied those that were unable to grow without glutamine. Not only did these mutants display very low GS activity, but the *glnA* transcript could not be detected when total RNA was probed. Further analysis of these mutants resulted in the identification of the *glnR* locus. It was found that the protein encoded by *glnR*, GlnR, is a DNA-binding protein which may either be directly involved in the activation of *glnA* transcription, or may indirectly control *glnA* expression by way of an intermediate regulatory gene

(Wray and Fisher, 1993). *C. glutamicum* was probed by Southern hybridisation with the *glnR* and *glnII* of *Streptomyces* to search for homologs of these genes.

At the time of this research (1998), adenylylation of the *C. glutamicum* GS had not been experimentally confirmed, and the *glnE* genes from only three different bacterial species were available in the National Centre for Biotechnology Information (NCBI) databases. These sequences included the *glnE* genes from *E. coli*, *M. tuberculosis* and *Haemophilus influenzae*. Based on deduced sequence analyses of the three genes, conserved regions were identified, and a PCR protocol was designed in order to amplify a region of the *C. glutamicum glnE* gene.

Various molecular methods are currently available that allow the screening of a bacterial genome for selected structural and regulatory sequences. Two of these techniques, transposon mutagenesis and promoter probe analyses, were employed in this study. The first reported isolation of a transposable element from a coryneform bacterium was by a Japanese group in 1994 (Vertés *et al.*, 1994a). They isolated the insertion sequence IS*31831* from *C. glutamicum* ATCC 31831. Although insertion sequences can be used to construct artificial transposons, the lack of a selectable marker makes them tedious to detect and isolate. In order to overcome this problem, Vertés and coworkers used the *B. subtilis sacB* gene which confers sucrose sensitivity to *C. glutamicum* (Jäger *et al.*, 1992). This gene allowed the development of a positive selection procedure for the entrapment of transposable elements in coryneform bacteria. The isolated element IS*31831* was subsequently used to construct two artificial transposons, Tn*31831* and mini-Tn*31831* (Vertés *et al.*, 1994b). The mini transposition vector efficiently mutagenized *Brevibacterium flavum* MJ233C at a rate of 4.3 x $10^4$ mutants per microgram DNA.

Promoter probing has been an equally successful molecular technique, and has been used extensively to study many *C. glutamicum* promoters (Pátek *et al.*, 1996; Reinsheid *et al.*, 1999; Wendisch *et al.*, 1997). This technique employs a plasmid that contains a promoterless selectable gene, such as antibiotic resistance. DNA fragments are then cloned upstream of the selectable gene and the resulting construct is then introduced

into the organism of interest. If the cloned fragment indeed displays promoter activity, it confers antibiotic resistance or some other selective phenotype to the host bacterium. In order to assess promoter strength in coryneform bacteria, Bernhard Eikmanns developed the promoter probe vectors pEKplCm (see Fig 2.1) and pEKpl*lacZ* (1991). Since both shuttle vectors are based on the replication origins of the corynebacterial pBL1 and the *E. coli* ColE1 plasmids, they are able to replicate in both *C. glutamicum* and *E. coli*. The pEKplCm vector carries the promoterless chloramphenicol-resistance gene (*cat*), while pEKpl*lacZ* carries the promoterless *lacZ* gene. The screening and identification of chloramphenicol-resistant or blue colonies allows the determination of promoter activity that can be further quantitated using chloramphenicol acetyltransferase (CAT) and β-galactosidase assays, respectively.

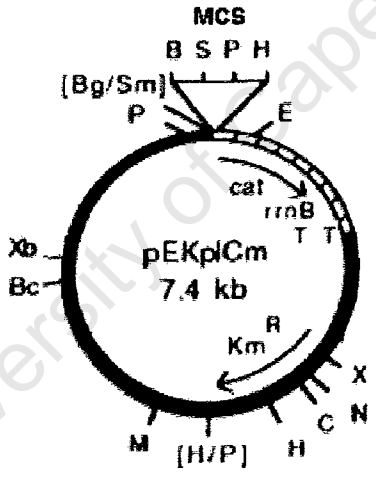



**Fig. 2.1** Diagram of pEKplCm. The multiple cloning site (MCS) has restriction sites for *BamHI* (B), *SalI* (S), *PstI* (P) and *HindIII* (H). (taken from Eikmanns *et al.*, 1991)

Thus, Southern hybridizations using heterologous probes, PCR, transposon mutagenesis and promoter probing techniques were employed in these studies with the aim of isolating novel nitrogen regulatory or assimilatory genes in *C. glutamicum*.

## 2.2 Methods

### 2.2.1 Bacteria, plasmids and media

The bacterial strains and plasmids used in this study are listed in Appendix A. Bacteria were routinely grown in LM (1% tryptone, 1% yeast extract, 0.5% NaCl, pH 7.2) at 37°C (*E. coli*) or 30°C (*C. glutamicum*) with aeration. For CAT assays, *C. glutamicum* strains were grown to mid-logarithmic phase in minimal medium (MM)(Siewe *et al.*, 1998) with nitrogen sources at the indicated concentrations. Antibiotics were added where appropriate. Nx and Km were both used at 50 µg/ml. Wild type *C. glutamicum* is inherently resistant to Nx (see Appendix A).

### 2.2.2 Preparation and manipulation of DNA

Plasmid DNA was extracted from *E. coli* using the Nucleobond® AX Kit (Machery-Nagel) according to the manufacturer's instructions or using the "High Pure Plasmid Isolation Kit" (Roche). *C. glutamicum* chromosomal and plasmid DNA were extracted according to the protocols described in Appendix B. For the purposes of plasmid restriction analysis, the "10 min" small-scale plasmid DNA extraction protocol was used to isolate plasmid DNA from *E. coli* (Appendix B). All enzymes for DNA manipulation and their respective buffers were obtained from Sigma, Roche or Amersham and used as instructed by the manufacturer. DNA manipulations were performed according to standard methods (Sambrook *et al.*, 1989).



### 2.2.3 Southern hybridizations

Restriction enzyme digests of the appropriate DNA were separated in a 0.8% agarose gel in Tris-acetate buffer. Southern blotting was performed as described by Sambrook *et al.* (1989) using Hybond-$N^+$ nylon membranes (Amersham). Homologous hybridizations: Hybridization (at 68°C), washing and detection procedures were performed according to the digoxigenin-11-dUTP (DIG) detection system of Roche Diagnostics (Germany). Heterologous hybridisations: As above, except that various hybridisation temperatures were used. Post-hybridization washes were performed under low stringency conditions using 2X SSC/0.01% SDS and 0.1X SSC/0.01 % SDS, where the 0.1X wash was performed at the hybridisation temperature. Stripping and reprobing

of membranes, as well as probe labelling by the random primed method, were also performed according to the DIG detection system of Roche. DIG-labelled probes used were: *C. glutamicum glnA* (~1kb *Stu*I-*Xba*I fragment of pGS-2/71), *S. coelicolor glnR* (~0.42kb *Sty*I and 0.27kb *Apa*I-*Sty*I fragments of pLEW146), *S. viridochromogenes glnII* (~0.55kb *Bgl*II-*Sal*I fragment of pDH19.2), *S. viridochromogenes glnA* (~0.75kb *Pst*I fragment of pDH18.1).

### 2.2.4 PCR

Reaction mixtures (50 µl) contained *C. glutamicum* genomic template DNA (200 ng), primers (0.6 µM each), dNTPs (50 µM each), $MgCl_2$ (2.0 mM), Red Hot™ Taq polymerase (2.5U; Advanced Biotechnologies) and its buffer, and were overlaid with sterile mineral oil. After an initial denaturation step (94°C, 3 min), the samples were subjected to 30 amplification cycles (1 min denaturation at 94°C, 1 min annealing at 52°C, 65s elongation at 72°C) using a JDI® high performance thermocycler. The following degenerate primers were used: $P_{AT1}$: 5' ATGGG(C/T)AAGTGCGG(C/T) 3', $P_{AT2}$: 5' (G/A)CCGCCCAGGC(G/T)(G/A)CC 3', where parentheses indicate base degeneracies. Primers were synthesized by Pei-Yin Ma (Dept. of Molecular and Cell Biology, Univ. of Cape Town, South Africa) using a Beckman Oligo 1000M DNA Synthesizer.



### 2.2.5 Cloning of PCR products

The desired PCR products were gel-purified using a Geneclean™ Kit (Bio 101, Inc.), and their ends were subsequently blunted and 5' phosphorylated. Purified PCR products were ligated with *Sma*I-digested, CIP-treated pBluescript SK. Ligation mixtures were transformed into competent *E. coli* DH5α cells (Dagert and Ehrlich, 1979).

### 2.2.6 Transposon mutagenesis

This protocol was obtained from Andreas Tauch (Dept. of Genetics, University of Bielefeld, Germany), and was specifically designed for conjugal transfer of the transposon delivery plasmid pTNC15. An O/N culture of the *E. coli* S17-1 (pTNC15) donor strain was prepared in 5ml LB (Sambrook *et al.*, 1989) with 2% glucose and antibiotic selection. The *C. glutamicum* RES167 recipient strain was grown O/N at

30°C in 2xLB to an optical density of at least 4.0. The donor strain was then diluted 1/100 into LB with 2% glucose, and grown to an optical density of 1.0 without antibiotic selection. A 10ml volume of *C. glutamicum* was heated at 48.5°C for 9 mins, after which 20ml of the donor was added to the recipient. The cells were mixed, pelleted by centrifugation (6000rpm, 5min, 30°C) and washed with LB. The bacterial pellet was then transferred to a cellulose acetate filter (Millipore, 0.45μm, diameter 47mm) which had been placed on a pre-warmed (38.5°C) LA plate. Once the filters were dry, the plates were incubated at 38.5 °C for 20 hrs. Cells were then washed off the filters in 5 ml LB, pelleted, and resuspended in 1ml LB. The mating mixture was plated on selective medium (LA with 2% glucose, 10μg/ml Cm and 50μg/ml Nx) and incubated at 30°C for 2-3 days.

### 2.2.7 Ampicillin enrichment

*C. glutamicum* transposon mutants (ca. $10^6$ cells) were added to 25ml LM and incubated at 30°C for 3h. Cells were subsequently pelleted by centrifugation (5000rpm, 5min, RT), washed twice with distilled water and resuspended in 1L MM containing 50μg/ml Ap. The culture was grown at 30°C for 6h after which the cells were again pelleted, washed twice with distilled water and resuspended in 2ml LM.

### 2.2.8 Promoter probe studies

Fragments 0.5–1.0kb in size were size-selected from a partial *Sau*3A digest of *C. glutamicum* chromosomal DNA and cloned into *BamH*I-digested, CIP-treated pEKplCm. The ligation mixture was then transformed into *E. coli* DH5α*mcr* and the transformants were grown up in bulk for a Nucleobond® plasmid extraction. The plasmid DNA was then introduced into *C. glutamicum* ATCC 13032 by electroporation (Appendix B) followed by selection on the appropriate medium.

### 2.2.9. CAT assays

*2.2.9.1 Preparation of cell free extracts*

*C. glutamicum* clones were grown O/N in MM containing 100mM $NH_4Cl$ and 50 μg/ml Km. Cells were then harvested by centrifugation (5,000rpm, 5min, RT), washed twice with TS buffer (50mM Tris-HCl, 50mM NaCl, pH 7.2) and resuspended in MM

containing the selected nitrogen source. Cell cultures were grown for 3 hours with shaking, after which cell free extracts (CFEs) were prepared. The cells were harvested by centrifugation, washed with 50mM Tris-HCl pH 7.6, and the pellet was resuspended in 1 ml of the same buffer. The suspension was sonicated (Virsonic; total processing time 6 min, 1 min sonication on highest power setting, 1 min cooling interval), and the cell debris was removed by centrifugation (14,000 rpm, 5 min, 4°C). CFEs were either used fresh (stored on ice) or frozen away.

*2.2.9.2 Assays*

CAT assays were performed according to a modified method of Shaw (1975). The reaction mixture contained 0.4mg/ml 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), 100mM Tris-HCl pH 7.8, 0.1 mM acetyl coenzyme A, and 0.1mM chloramphenicol. One unit CAT activity = 1μmole of chloramphenicol acetylated per minute. Specific CAT activity was expressed as units per mg protein in the cell fee extract. Protein concentrations were determined by the dye-binding method (Bradford, 1976). Activities displayed in the results represent the average of three independent assays which differed by no more than 20%.



**2.2.10 Nucleotide sequencing**

Cloned DNA fragments were sequenced using the dideoxy nucleotide triphosphate chain-termination method of Sanger *et al.* (1977). Cycle sequencing reactions were performed using the Thermosequenase™ cycle sequencing kit (Amersham) and a Hybaid Ominigene thermocycler. Bidirectional sequencing of fragments cloned into Bluescript SK was performed using fluorescent end-labeled Cy5-Far Red primers (fwd 5'CGCCAGGGTTTTCCCGTCACGAC 3', rev 5' GAGCGGATAACAATTTCACAC-AGG 3'). Partial sequencing of inserts cloned into pEKplCm was performed using a Cy5-labeled primer CmS (5' GGTGGTATATCCAGTGAT 3') which covers positions +30 to +13 relative to the start of transcription of the *cat* gene (Pátek *et al.*, 1996). Sequencing products were subsequently run on an ALF-Express™ automated sequencer (Pharmacia Biolabs) as instructed by the manufacturer. AM Version 3.02 computer software was used to interpret and manipulate the information derived from the ALF-Express. The nucleotide sequences were analysed on a VAX 6000-330 computer using the Genetics Computer Group program (Devereux *et al.*, 1984).

## 2.3 Results

### 2.3.1 Southern hybridization with heterologous probes

Southern hybridization studies were used to determine whether *C. glutamicum* has genes that encode homologs of GSII and GlnR. *C. glutamicum* chromosomal DNA was extracted, digested with *Bam*HI endonuclease, and used for Southern hybridization with various probes. The heterologous probes used included fragments of genes from *Streptomyces coelicolor glnR*, *Streptomyces viridochromogenes glnII*, and *S. viridochromogenes glnA*.

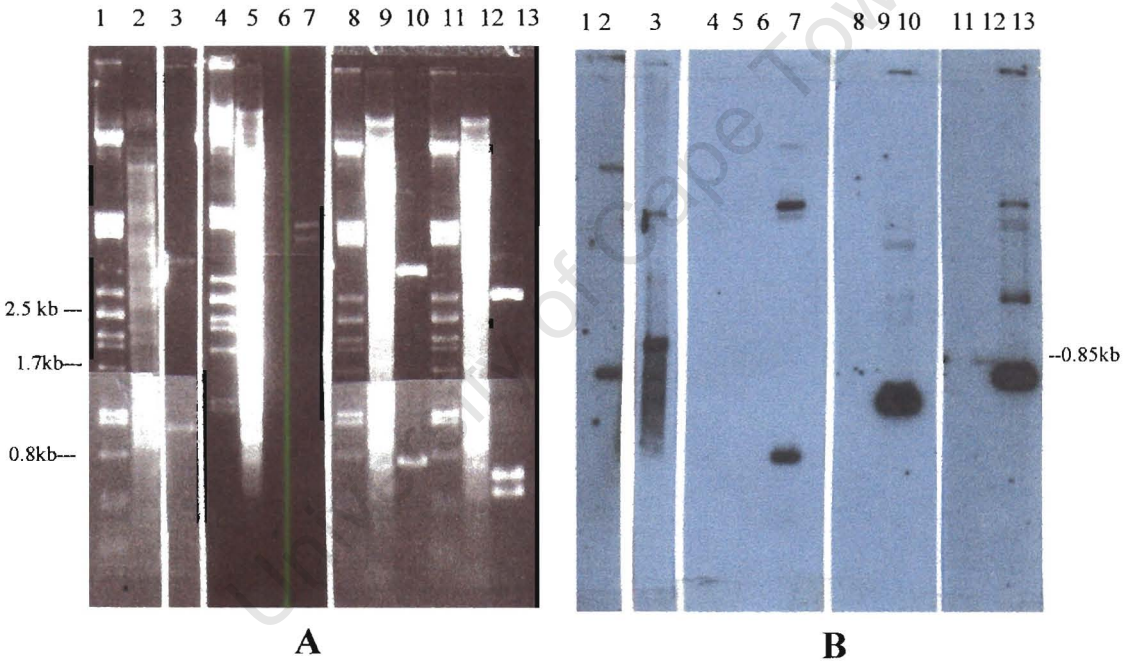



**Fig. 2.2.** Heterologous Southern hybridizations at 50°C to search for *glnR* and *glnII* homologs in *C. glutamicum*. **A:** Agarose gel electrophoresis of endonuclease-digested DNA. Lanes 1,4,8,11: λ-*Pst*I molecular markers; lanes 2,5,9,12: *C. glutamicum* chromosomal (35 µg), *BamH*I; lane 3: pGS-2/71, *Stu*I-*Xba*I; lane 7: pLEW146, *Sty*I; lane 10: pDH18.1, *Pst*I; lane13: pDH19.2, *Bgl*II-*Sal*I. **B:** Corresponding autoradiograph hybridized with the following probes. Lanes 1-3: *C. glutamicum glnA* probe; lanes 4-7: *S. coelicolor glnR* probe; lanes 8-10: *S.viridochromogenes glnII* probe; lanes 11-13*: S. viridochromogenes glnA* probe.

At a hybridization temperature of 50°C, the *Streptomyces glnA* probe hybridized to a 0.85 kb fragment of the *Bam*HI-restricted *C. glutamicum* chromosome (Fig. 2.2, lane B12). As expected, a 0.85 kb fragment was also detected by the *C. glutamicum glnA* probe (Fig. 2.2, lane B2). However, no hybridization occurred between the *glnII* and

*glnR* probes and the *C. glutamicum* chromosome at this temperature (Fig. 2.2, lanes B5 & B9). The heterologous hybridization was therefore repeated at lower temperatures. The hybridization temperature was sequentially decreased, until a minimum of 40°C was used. However, even at this low stringency, no *C. glutamicum* chromosomal fragments hybridized with the *glnR* and *glnII* probes (Fig. 2.3, lanes B4 & B5).

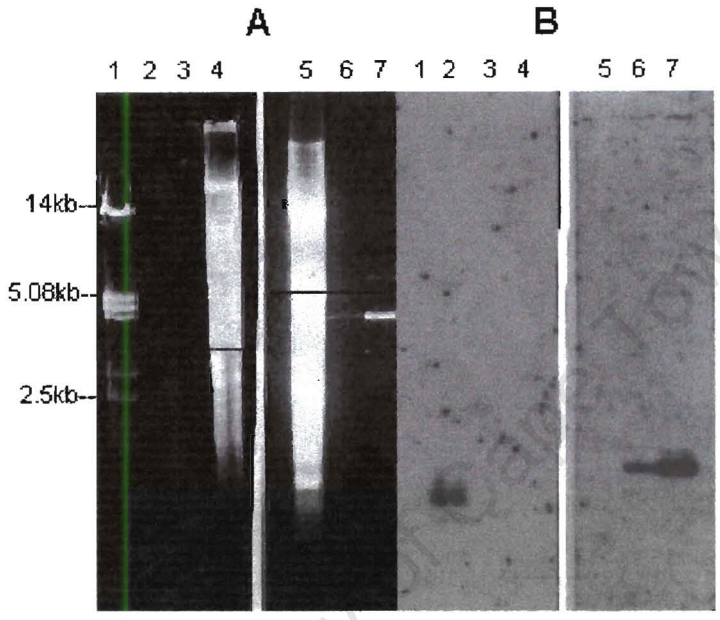



**Fig. 2.3.** Heterologous Southern hybridizations at 40°C to search for *Streptomyces glnR* and *glnII* homologs in *C. glutamicum* **A**: Agarose gel electrophoresis of endonuclease-digested DNA. Lane1: λ-*Pst*I molecular marker; lanes 4, 5: *C. glutamicum* chromosomal (35 µg), *Bam*HI; lane 2: pLEW146, *Apa*I-*Sty*I; lane 7: pDH19.2, *Sal*I-*Bgl*II. **B**: Corresponding autoradiographs of hybridization with the following probes. Lanes 1-4: *S. coelicolor glnR* probe; lanes 4-7: *S. viridochromogenes glnII* probe.



### 2.3.2 Attempted PCR amplification of the *C. glutamicum glnE* gene

The deduced amino acid sequences of the GlnE proteins from *E. coli, H. influenzae*, and *M. tuberculosis* were aligned using the Genetics Computer Group program (Devereux *et al.*, 1984), and several conserved regions were identified. Degenerate oligonucleotide primers were designed based on two of these conserved regions (Fig. 2.4) and *C. glutamicum* codon preference.

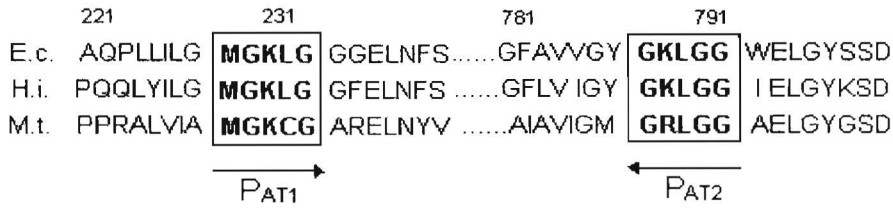



**Fig. 2.4.** Partial amino acid aligmment of GlnE proteins from 3 different bacteria. Regions used to design degenerate primers ($P_{AT1}$ & $P_{AT2}$) are boxed and bolded. E.c.: *E.coli* (Acc. No. NP_289628); H.i.: *H. influenzae* (Acc. No. AAC21747); M. t.: *M. tuberculosis* (Acc. No. CAA94664). *E. coli* amino acids are numbered.

The designed primers were intended to amplify an internal region of the putative *glnE* gene from the *C. glutamicum* chromosome by PCR. At an annealing temperature (Ta) of 52°C, three major consistent with the expected size range were amplified (Fig. 2.5).

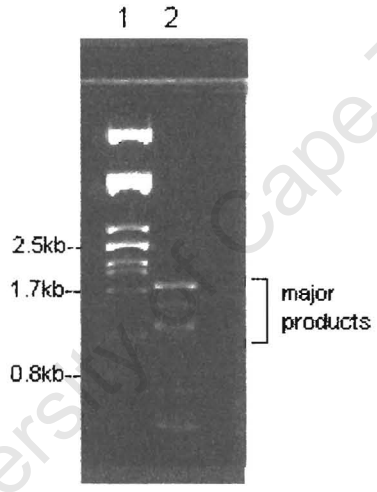





**Fig. 2.5** Agarose gel electrophoresis of PCR products obtained using primers designed to amplify an internal region of a putative *C. glutamicum glnE* gene. Lane 1: λ-*Pst*I molecular marker; lane 2: PCR products (Ta=52°C).

Since the exact size of the desired PCR product was unknown, all three products were isolated and cloned. Sequencing of the PCR products and subsequent BLAST analyses using the NCBI databases (www.ncbi.nih.nlm.gov), revealed that none of them displayed significant homology to one another or previously isolated *glnE* sequences. Lowering of the annealing temperature, and repeated attempts at PCR using a hotstart method and 10% DMSO, resulted in amplification of the same three products. Elevating the annealing temperature simply increased the yield of the largest PCR product.

### 2.3.3 Transposon mutagenesis

Initial attempts at mutant generation involved electroporation of *C. glutamicum* RES167 (Appendix B). This method, however, proved unsuccessful, and conjugal transfer of pTNC15 from the *E. coli* S17-1 donor to *C. glutamicum* RES167 was therefore used. Repeated mating experiments with slight variations in the protocol had to be performed to optimize the procedure. Transconjugants were obtained at a frequency of $10^{-6}$ to$10^{-3}$ per donor cells such that between $10^3$ and $10^6$ mutants were generated per mating experiment. An ampicillin enrichment was subsequently performed in order to select for auxotrophic mutants. The enriched culture was plated onto LA with 10μg/ml Cm and 50μg/ml Nx, and 43 colonies were obtained in total. These colonies were replica plated onto MM containing the same antibiotics and with or without 20mM glutamine. Surprisingly, all colonies grew on the MM, indicating that these were not auxotrophic mutants. To investigate this unexpected result, six clones were randomly selected from the MM plates and were grown up for chromosomal extractions.



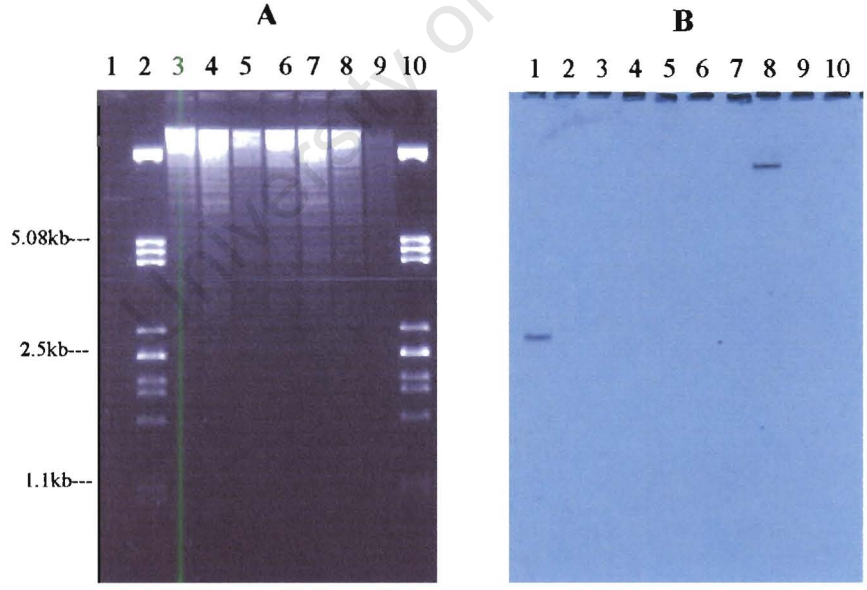



**Fig. 2.6** Southern hybridisations to confirm Tn*5515* integration into the chromosomes of several putative *C. glutamicum* mutants. **A**: Agarose gel electrophoresis of endonuclease-digested DNA. Lane 1: 10ng pTNC15; lanes 2 & 10: λ-*Pst*I molecular marker; lanes 3-8: *Xba*I-digested chromosomal DNA from putative transposon mutants; lane 9: *Xba*I-digested wild type *C. glutamicum* chromosomal DNA. **B**: Corresponding autoradiograph of hybridization with the ~1.9kb *Xba*I fragment of pTNC15.

Their DNA was used for Southern hybridisations with a Tn*5515*-specific probe (Fig. 2.6). This analysis indicated that only one of the six putative *C. glutamicum* transposon mutants had Tn*5515* integrated into the chromosome.

In parallel with the above studies, we also screened existing pools of transposon mutants of *C. glutamicum* ATCC 14752 obtained from Andreas Burkovski (Univ. of Cologne, Germany). This *C. glutamicum* strain is very similar to ATCC 13032, and the mutants had been generated using the transposon delivery plasmid pCGL0040 (carrying Tn*5531*). Four pools were screened, and each pool contained approximately 500 different mutants. Each pool was diluted and plated onto BHI agar plates containing Km, Nx and 5mM glutamine. The colonies (ca. 5.5 x $10^2$ in total) were subsequently replica plated onto MM (Appendix B) with Km and Nx. Most colonies grew well on the MM plates, however, 121 of them seemed to display reduced growth. The mutants were re-streaked several times onto MM and MM with 20mM glutamine plates. However, after extensive growth studies, none of these mutants displayed the original reduced growth in the absence of glutamine.

Since we were unable to isolate any desired *C. glutamicum* mutants by transposon mutagenesis, we decided to employ promoter probing as an alternative technique for isolating nitrogen metabolic and/or regulatory genes.

### 2.3.4. Promoter probe studies

In order to identify promoter sequences responsive to nitrogen status, a *C. glutamicum* promoter probe genebank was created in the promoterless *cat* vector pEKplCm. This genebank was electroporated into *C. glutamicum* ATCC 13032, and several electroporation experiments were pooled to yield a total of ca. 4 x$10^3$ transformants. Several of these Km–resistant colonies were randomly selected and their plasmid DNA was subjected to restriction analysis. All colonies harboured plasmids with inserts of the expected size range (results not shown).

Transformants were plated onto 2mM $NH_4Cl$ MM containing 5μg/ml Cm and then replica plated onto 100mM $NH_4Cl$ MM also containing 5μg/ml Cm. Of these transformants, 62 colonies grew better on low than high nitrogen, and were therefore replated in the same way several times. Finally, 8 clones (designated #1 to #8) remained that displayed enhanced Cm resistance on 2mM $NH_4Cl$ MM in comparison to 100mM $NH_4Cl$ MM. The promoter probe constructs harboured by these clones were designated #1-CAT to #8-CAT. The minimum inhibitory concentration (MIC) for Cm was determined in liquid medium and on plates, and CAT activity assays were also performed on these clones grown in 2mM and 100mM $NH_4Cl$ MM. As controls, the *C. glutamicum glnA* promoter was cloned into pEKplCm and introduced into *C. glutamicum*, and a random Cm-resistant clone with promoter activity was selected. The constructs of these clones were designated pGS-CAT and nonN-CAT, respectively. *C. glutamicum* containing pEKplCm served as a negative control. In order to further characterize the cloned sequences, the plasmid inserts of these clones were partially sequenced. Sequences were subjected to BLAST analyses within the NCBI databases (www.ncbi.nih.nlm.gov) to search for previously identified protein homologs. Sequencing results revealed that some isolated fragments were indeed involved in nitrogen metabolism, and one of the constructs was found to contain the randomly isolated *glnA* promoter. All the abovementioned results are summarized in Table 2.1. Interestingly, Cm resistance levels differed depending on the nature of the medium and were not directly correlated with CAT activity.

Since sequencing of #5-CAT and #8-CAT revealed that they contained promoter sequences with homologies to hypothetical proteins, and clones #5 and #8 showed a marginal increase in Cm resistance under low nitrogen (Table 2.1), these two clones were investigated further. Both clones, as well those containing pGS-CAT and nonN-CAT, were grown in MM with various nitrogen sources, and their activity profiles were compared.

| Plasmid Construct | Cm MIC High N | | Cm MIC Low N | | CAT activity (U/mg protein) | | BLAST result (protein) | E value |
|---|---|---|---|---|---|---|---|---|
| | L | P | L | P | High N | Low N | | |
| No promoter-CAT | <1 | | <1 | | 0.004 | 0.001 | N/A | |
| #1-CAT | 10 | 25 | 10 | 30 | 0.34 | 0.33 | *M.l.* cystathione gamma synthase[1] | $10^{-31}$ |
| #2-CAT | 15 | 20 | 15 | 25 | 0.24 | 0.15 | *P.v.* mutator MutT protein[2] | $10^{-11}$ |
| #3-CAT | 15 | 20 | 25 | 30 | 0.41 | 1.11 | *M.t.* glutamine synthetase I[3] | $10^{-3}$* |
| #4-CAT | 25 | 25 | 20 | 25 | 0.48 | 0.84 | *B.s.* methionine aminopeptidase[4] | $10^{-7}$ |
| #5-CAT | 20 | 20 | 20 | 20 | 0.22 | 0.28 | *M.t.* hypothetical 14 kD protein[5] | $10^{-7}$ |
| #6-CAT | 10 | 25 | 25 | 30 | 0.32 | 0.19 | *T.t.* electron transfer flavoprotein[6] | $10^{-17}$ |
| #7-CAT | 25 | 25 | 25 | 25 | 0.43 | 0.22 | *M.t.* histidyl tRNA synthetase[7] | $10^{-50}$ |
| #8-CAT | 30 | 25 | 45 | 30 | 0.15 | 0.20 | *E.c.* 26.4 kD hypothetical protein[8] | $10^{-13}$ |
| pGS-CAT | 25 | 25 | 35 | 30 | 0.47 | 1.06 | *H.s.* zinc finger protein[9] | 2.1* |
| NonN-CAT | 35 | 30 | 45 | 30 | 1.21 | 0.75 | *M.t.* 50S ribosomal protein L14[10] | $10^{-2}$ |

**Table 2.1** CAT activity and identity of selected promoter probe constructs in *C. glutamicum* containing *C. glutamicum* chromosomal fragments. *M.l.*: *Mycobacterium leprae; M.t. Mycobacterium tuberculosis*; *P.v.*: *Proteus vulgaris*; *B.s.*: *Bacillus subtilis*; *T.t.*: *Thermoaerobacterium thermosaccharolyticum*; *E.c.*: *Eschericia coli*; *H.s.*: *Homosapiens sapiens*. 1. Acc. No. NP_302550, 2. Acc. No. P32090, 3. Acc. No. Q10377, 4. Acc. No. NP_388019, 5. Acc. No. NC_000962, 6. Acc. No. Z92974, 7. Acc. No. NC_000962, 8. Acc. No. NC_002655, 9. Acc. No. BC026092, 10. Acc. No. NC_000962. High N: 100mM $NH_4Cl$, Low N: 2mM $NH_4Cl$. L: liquid, P: plate * E value = 0.0 for *C. glutamicum glnA* gene at the nucleotide level. MICs are in μg/ml.

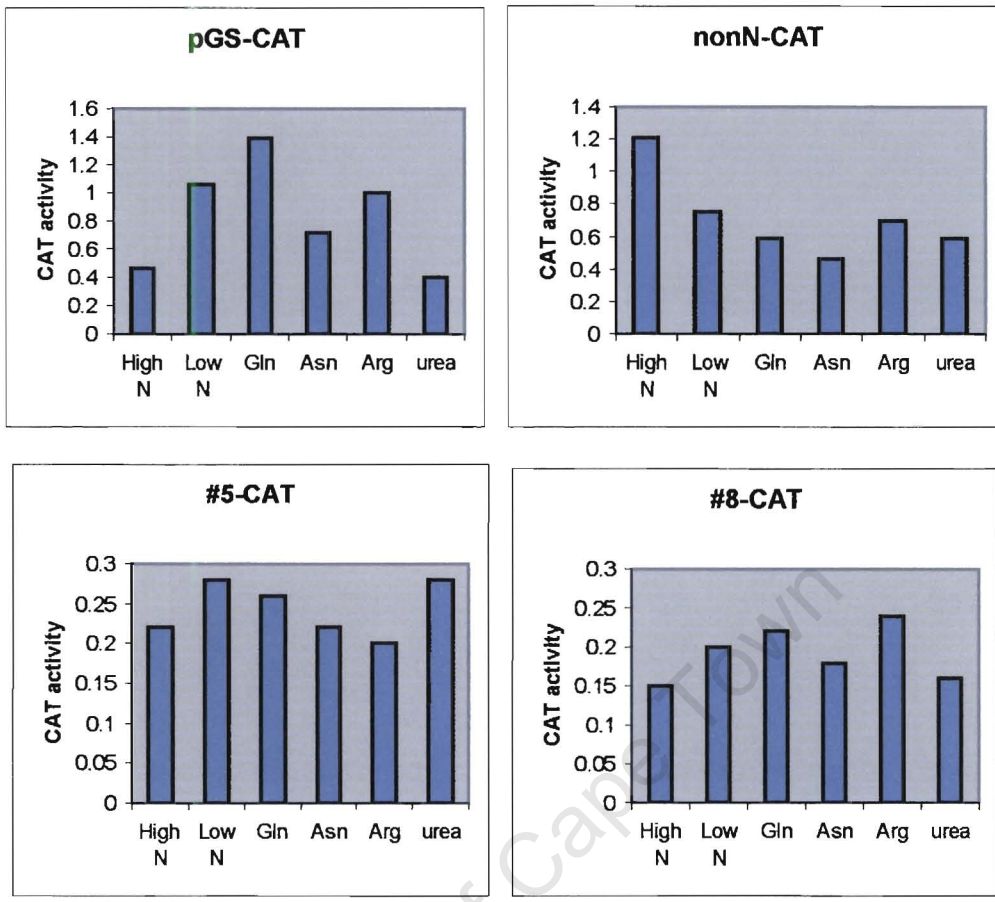



**Fig. 2.7** CAT activities of selected promoter probe constructs in *C. glutamicum* grown in MM with various nitrogen sources. Gln: glutamine, Asn: asparagine, Arg: arginine, High N: 100mM $NH_4Cl$, Low N: 2mM $NH_4Cl$. Amino acids were used at 20mM and urea at 2%. Activities are the means of at least 2 independent experiments that did not differ from each other by more than 20%. High and low N results are taken from Table 2.1.



As seen in Fig. 2.7, the *glnA* promoter of pGS-CAT is induced not only by low $NH_4Cl$, as shown previously, but also by glutamine, arginine and asparagine to a lesser extent. Urea has the same effect on the control promoter as high $NH_4Cl$ conditions. Clones #5 and #8, however, show only slight induction in low nitrogen, glutamine, asparagine and arginine. In contrast, CAT activity of the clone containing nonN-CAT is essentially unaffected by the nature of the nitrogen source but is highest in 100mM $NH_4Cl$ conditions. Thus, clones #5 and #8 have similar CAT activity profiles as the clone containing pGS-CAT i.e. lowest activity in high nitrogen. However, the induction by the indicated nitrogen sources was not even two-fold.

The subsequent sequencing of the complete *C. glutamicum* genome (2001) enabled us to determine more precisely what sequences were contained by #5-CAT and #8-CAT. The insert of #5-CAT was mapped to an unknown *C. glutamicum* sequence which has a hypothetical *C. glutamicum* gene upstream named ORF1. The insert of #8-CAT, on the other hand, mapped to the *C. glutamicum yfcA* gene, which is a hypothetical structural protein. This gene is flanked by sequences that encode acyl-CoA thiolesterase II and the crossover junction endodeoxyribonuclease, RuvC. Since the sequencing data, in combination with the assays, provided no substantial clues as to the role of these genes, they were thus not investigated any further.

## 2.4 Discussion

Various molecular techniques were employed to identify gene homologs and novel genes involved in nitrogen metabolism and regulation in *C. glutamicum*. Southern hybridization experiments with heterologous probes revealed that the *S. viridochromogenes glnA* probe was able to detect the same 0.85kb fragment in the *C. glutamicum* chromosome as the homologous *glnA* probe. This result once again underscores the high degree of conservation between GSI proteins, in particular those of *Corynebacterium* and *Streptomyces* (Jakoby *et al.*, 1997). Probing with the *Streptomyces glnR* and *glnII* genes, however, did not result in the identification of similar sequences in the *C. glutamicum* genome. The *glnII* probe used in our study was ca. 550bp in size and consisted of almost half of the gene. This suggests that either no *glnII* gene is present in *C. glutamicum*, or our probe was not sufficiently homologous in the selected region. A recent discovery by Nolden and coworkers (2001) indicated that the latter was the case. This group was able to identify a second GS gene in *C. glutamicum*, which they called *glnA2*. The deduced amino acid sequence of this gene shows high similarity to that of the *M. tuberculosis* GSII (65%), but only has weak similarity to the *C. glutamicum glnA*. Further characterisation of *glnA2* revealed that the gene is constitutively expressed and independent of nitrogen supply. Deletion of *glnA2* resulted in no growth defects in the mutants under nitrogen limitation and cellular GS activity was unaffected. The physiological role of *glnA2*, therefore, remains unclear. Our findings further corroborate the fact that this *glnA2* gene is unlikely to play a significant role in glutamine synthesis, since *C. glutamicum glnA* deletion mutants (generated in this research) are auxotrophic for glutamine and do not display any GS activity (discussed in Chapter 4). This is in contrast to *S. viridochromogenes*, where neither *glnA* nor *glnII* mutants require glutamine for growth, suggesting that either GS can synthesize glutamine and compensate for lack of the other (Hilleman *et al.*, 1993).

In the case of *glnR*, a 420bp heterologous probe internal to the gene was used in the initial hybridization experiments. At the lowest hybridization temperature of 40°C, however, no hybridization signal was obtained when both the 420bp and 270bp probes (which represent most of the 801bp *glnR* gene) were used together. Therefore, under

these conditions, any sequence with significant homology would most likely have been detected. Thus, it seems unlikely that *C. glutamicum* harbours a sequence homolog of *glnR*. The deduced amino acid sequence of the *S. coelicolor glnR* gene is similar to that of several bacterial response regulators that are known to function as transcriptional activators (Wray and Fisher, 1993). The GlnR protein belongs to the subgroup which contains OmpR, PhoB, VirG and ArcA. Both VirG and OmpR proteins have been shown to have a DNA-binding domains at their C-terminal ends. In *S. coelicolor*, the *glnA* promoter is similar to other positively-regulated promoters in that it has significant homology with the –10 consensus sequence but not with the –35 consensus sequence for vegetative *Streptomyces* promoters (Fisher and Wray, 1989). Thus, it seems that GlnR is a DNA-binding protein that is directly responsible for the activation of *glnA* expression. Similarly, we find the same promoter structure in the *glnA* of *C. glutamicum* (see Chapter 3) where the –10 region displays significant homology to the consensus sequence of other *C. glutamicum* promoters, while the –35 region is less conforming. Furthermore, the same promoter is employed by the *C. glutamicum glnA* gene even when transcription is enhanced (see Chapter 3), thus control of transcription is very likely to involve a protein factor. As mentioned previously, the AmtR protein binds upstream of *glnA* and causes repression of transcription during nitrogen excess; this repression is lifted when nitrogen becomes limiting (Nolden *et al.*, 2001). The involvement of a repression rather than an activation protein in the control of *glnA* expression corroborates the negative result for the *glnR* heterologous hybridizations.

Attempts at amplifying a *C. glutamicum glnE* homolog by PCR were unsuccessful in our studies. Subsequent to this research, Fink and colleagues (1999) were able to amplify the *S. coelicolor glnE* gene using the same method, although they based their primer design on different conserved regions and the appropriate codon preference. Hybridization analyses performed by Fink *et al.* (1999) revealed that the *glnE* gene is not a unique feature of *S. coelicolor*, and all 18 of the *Streptomyces* strains that were studied harboured the *glnE* gene. The putative functional adenylyltransferase domain shows a high degree of conservation in the GlnE proteins (Fink *et al.*, 1999). The reverse primer used for the PCR performed in the research described here was based on the amino acid sequence GRLGG which is highly conserved within this domain.

Changing the PCR conditions such as template and $Mg^{2+}$concentrations only affected the relative quantities of the three major products, as did modifications specifically used for high G+C DNA such as hotstart PCR and DMSO addition. Presumably, the forward primer was not sufficiently specific for the *glnE* gene. Recently, and subsequent to our PCR studies, the entire *C. glutamicum* genome was sequenced, and sequence analysis revealed that a *glnE* was indeed present in this organism. This gene forms part of an operon with *glnA2*, and, interestingly, transcription of *glnA2-glnE* is constitutive and independent of nitrogen status (Nolden *et al.*, 2001). Gene knockout studies by Nolden and coworkers confirmed the role of this *glnE* gene as coding for the funtional adenylyltransferase enzyme.

Screening transposon banks for *C. glutamicum* mutants affected in nitrogen metabolism posed several problems. Previous experiments in our laboratory using the *E. coli-C. glutamicum* shuttle vector pWK0 have shown that *C. glutamicum* can be electroporated at an efficiency of $10^6$ transformants/μg DNA. This efficiency is in the same order of magnitude as those routinely achieved by Liebl *et al.* (1989), although both the plasmid and the *C. glutamicum* strain used were different. Thus, the introduction of pTNC15 into this bacterium by means of electroporation should have been highly efficient. However, assessing such efficiency was not possible since pTNC15 is a suicide vector. It was subsequently revealed that due to the replicative transposition mechanism of *IS*1249, the insertion sequence of pTNC15, transposon insertion and subsequent mutagenesis is dependent upon transconjugation (Andreas Tauch, Univ. of Bielefeld, Germany, personal communication). This is in contrast to the insertion sequence previously described by Vertés *et al.* (1994), where the delivery plasmid was simply electroporated into *C. glutamicum* and resulted in the generation of mutants.

RP4-mediated transfer of mobilizable plasmids from *E. coli* donors to *C. glutamicum* by conjugation is hampered by restriction systems in the recipient (Schäfer *et al.*, 1994a). Fortunately, restriction-deficient mutants of *C. glutamicum* are available and certain environmental stresses have been shown to impair the restriction system (Schäfer *et al.*, 1994b). In this study, the recipient cells were heated to 48.5°C for 9 minutes, conditions which have been shown to greatly increase mating frequencies (Schäfer *et al.*, 1994b).

For transposon mutagenesis, the use of the restriction-deficient strain *C. glutamicum* RES167 in combination with heat treatment resulted in the generation of transconjugants at a high frequency of between $10^{-3}$ and $10^{-6}$ per donor cell. These frequencies are in the same order of magnitude as those obtained by Andreas Tauch (Univ. of Bielefeld, Germany, personal communication). Despite the high conjugation frequencies, no auxotrophic mutants could be isolated in our studies. In fact, of six putative *C. glutamicum* transposon mutants randomly selected after ampicillin enrichment, five of them did not have Tn*5515* integrated into their chromosome. This was most likely due to excision of the transposon during stressful growth in the ampicillin enrichment. Furthermore, subsequent communication with Andreas Burkovski (University of Cologne, Germany) revealed that Tn*5515*, the transposable element in pTNC15, is not as random as initially thought, and has a preference for AT-rich regions. This fact might explain why no auxotrophic mutants were generated, since the coding regions of *C. glutamicum* are G-C rich. It is important to note that our screening media was very selective and would only identify mutants that had impaired glutamine synthesis. With regards to the *C. glutamicum* Tn*5531* mutants that we obtained from Burkovski, the relatively small size of the transposon genebank in combination with the highly selective media could explain why no desired mutants were isolated.

In similar studies, researchers have used transposons successfully to identifying nitrogen-regulated sequences. Atkinson and Fisher (1991) created a library of Tn*917-lacZ* insertions in *B. subtilis* and screened it for β-galactosidase activity in response to ammonium limitation. This approach allows convenient selection as well as the accurate quantitation of expression. Although no analogous Tn-*lacZ* construct is available for *C. glutamicum*, we decided to utilise a similar selectable technique to identify nitrogen-responsive sequences, namely promoter probing.

Our promoter probe genebank was generated in the vector pEKplCm rather than in pEKpl*lacZ* since the distinction between blue and white colonies, the selection basis for pEKpl*lacZ*, often becomes difficult on certain media. Antibiotic resistance selection, on the other hand, is generally more clear-cut. Introduction of our genebank constructs

into *C. glutamicum* occurred at a low frequency due to the large size of the plasmids (up to 8.5 kb), and thus the transformants from several electroporation experiments were pooled. Once the genebank was introduced into *C. glutamicum*, colonies that appeared to display enhanced growth under low $NH_4Cl$ were selected. Many rounds of restreaking were required to confirm this phenotype, and even then, the selection of colonies that grew better under low $NH_4CL$ was rather subjective. This was, in part, due to the fact that *C. glutamicum* cells grow much more vigorously on high $NH_4Cl$ MM than low $NH_4Cl$ MM. Also, the compounded environmental stress of the MM, low nitrogen and Cm may retard cell growth and mask enhanced antibiotic resistance to some extent. Therefore, the only effective method of determining whether a specific promoter probe was truly induced by low $NH_4Cl$ required the use of CAT assays. The 8 clones that seemed to respond to nitrogen status were subjected to CAT assays after growth in high and low $NH_4Cl$ MM. MIC determinations for Cm were also performed on these clones in the same media.

The finding that the relationship between Cm MICs and CAT activity was not linear has been observed previously in *C. glutamicum*, as well as in *E. coli* and *B. subtilis* (Pátek *et al.*, 1996). The reason for this remains unclear. This finding, however, highlights the limited application of this technique when selecting promoters on Cm plates. For example, the CAT activity of the clone containing the control GS promoter construct is around two-fold higher under low $NH_4Cl$ conditions than high $NH_4Cl$ conditions, whereas the Cm MIC of the same clone is only slightly higher on low $NH_4Cl$ plates than high $NH_4Cl$ plates (Table 2.1). Eikmanns *et al.* (1991) showed that the copy number of pEKplCm and promoter-pEKplCm constructs were very similar, thus ruling out major errors in the evaluation of promoter strength due to different reporter gene doses. An additional limitation of promoter probing is the cloning of promoters that, although normally responsive to nitrogen status, may not be active or induced/repressed due to sequence truncations and/or the absence of essential promoter elements. Also, the presence of a sequence that binds proteins on the multicopy promoter-probe plasmid may result in the titration of certain regulatory proteins, thus affecting the levels of induction or repression that would occur under normal *in vivo* conditions (see pGS-CAT activity, discussed below). Nonetheless, and despite these limitations, several promoters

of genes involved in nitrogen metabolism were isolated in our studies, the most noteworthy of these being the *glnA* promoter. Nucleotide sequencing showed that this *glnA* promoter fragment contained more of the flanking sequences than the promoter of the control pGS-CAT construct, thus confirming the authenticity of this promoter probe isolate from the genebank. The other promoter sequences belong to genes which encode proteins similar to others in the database that are involved in nitrogen metabolism. These are cystathione gamma synthase, methionine aminopeptidase, and histidyl tRNA synthase. The former two proteins are involved in methionine metabolic pathways and the latter in the synthesis of histidyl tRNA molecules. The remaining sequences belong to putative proteins similar to the mutator MutT protein, which is involved in DNA repair, and the electron transfer flavoprotein which is involved in cellular energy processes.

In yet another approach, Schmid *et al.* (2000) used two-dimensional protein gel electrophoresis coupled with microsequencing to examine *C. glutamicum* proteins that were synthesized in response to nitrogen starvation. Using this technique they were able to identify 15 such proteins. Many of them were unknown proteins, however, several of them were enzymes of central carbon metabolism while others were involved in protein synthesis, as was the case with two of the proteins isolated by promoter probing described here. Unexpectedly, no proteins known to be induced by nitrogen deprivation were identified in Schmid's studies. They suggest that this may be the result of low expression rates and/or the short exposure time (only 40 min) in the inducing conditions.

Constructs #5-CAT and #8-CAT were found to contain promoter sequences of genes encoding hypothetical proteins. To establish whether these genes might be novel and involved in nitrogen metabolism, CAT activity profiles on various nitrogen sources were compared with those of pGS-CAT and nonN-CAT. The activity profiles showed that the CAT activities of both #5 and #8 were similar to the control GS promoter that showed enhanced CAT activity in response to glutamine, arginine and asparagine. The rates of induction of #5 and #8 were, however, much lower than in the case of the GS promoter. Thus, whether the promoter sequences contained by #5-CAT and #8-CAT

may in fact be nitrogen-regulated and/or involved in nitrogen metabolism is rather speculative, but cannot be completely ruled out.

Induction of the *glnA* promoter of pGS-CAT by low $NH_4Cl$ is only two-fold in these promoter probe studies, while transcription of chromosomal *glnA* in *C. glutamicum* is around 5-fold under the same conditions (see section 3.3.4). This significant difference suggests the requirement of additional sequences or regulatory proteins in the control of *glnA* transcription. Considering the role of AmtR in *glnA* repression (Nolden *et al.*, 2001), this protein may be unable to effect sufficient repression of transcription from the *glnA* promoter probe construct due to the copy number of the expression vector. Given this scenario, relative *glnA* promoter probe induction levels would appear reduced in comparison to the repressed condition because the repressed expression level is higher than normal. Although the copy number of pEKplCm has never been precisely determined, it is estimated to be between 10 and 30 copies per cell (Bernhard Eikmanns, University of Ulm, Germany, personal communication). This is consistent with our plasmid extraction yields which suggest that this vector does not have a low copy number. In contrast to our findings, promoter probe studies performed by Reinsheid *et al.* (1999) and Wendisch *et al.* (1997) using pEKplCm showed that promoter induction levels were consistent with Northern hybridization experiments.

Ammonium and urea are preferred sources of nitrogen for *C. glutamicum* (Siewe *et al.*, 1998), which explains why CAT activities of the pGS-CAT construct were lowest under these conditions. During nitrogen deprivation, ammonium uptake in *C. glutamicum* is mediated by the product of the *amt* gene (Siewe *et al.*, 1996). In the case of urea, this compound passively diffuses into the cell when nitrogen is sufficient, while an energy-dependent urea-uptake system is synthesized when nitrogen is in short supply (Siewe *et al.*, 1998). Once inside the cell, the urease enzyme releases ammonia from urea, and the activity of this enzyme is highest under nitrogen sufficiency. Asparagine and arginine are less favoured nitrogen sources compared to urea and ammonium, which may explain the relative induction of the *glnA* promoter of pGS-CAT. Once inside the bacterial cell, both arginine and asparagine are catabolized to yield glutamate (Voet and Voet, 1990). The significant induction of this promoter in response to glutamine may seem rather

surprising since it seems counterproductive in terms of cellular metabolism. Glutamine is considered a good source of nitrogen for *C. glutamicum* (see section 4.3.4), and uptake of this amino acid occurs via a sodium-dependent symport mechanism (Siewe *et* al., 1995). The MM used in our studies had sufficient sodium ions present (>20mM) to allow the efficient uptake of glutamine. Therefore, the control *glnA* promoter construct behaves as predicted in response to some nitrogen sources, but unexpectedly in the case of others. It is likely that the copy number of pGS-CAT affects the cellular AmtR repression mechanism, which in turn results in the variable regulation of the *glnA* promoter on the construct.

Since no novel nitrogen regulatory genes could be identified by Southern hybridization, transposon mutagenesis or promoter probing, we decided to focus on the regulation of GS and GOGAT activity in *C. glutamicum*.

# Chapter 3

## Regulation of Glutamine Synthetase and Glutamate Synthase in *C. glutamicum*


### Abstract

The effect of nitrogen and carbon status on the regulation of glutamine synthetase (GS) and glutamate synthase (GOGAT) were investigated in *C. glutamicum*. When carbon was sufficient (2% glucose) and nitrogen limiting (2mM $NH_4Cl$), GS and GOGAT activities were 5- and 7-fold higher, respectively, in comparison to high nitrogen conditions (100mM $NH_4Cl$). GS activity was also induced when cells were grown in complete medium with 1% added glucose, while GOGAT activity was unaffected. Northern hybridizations revealed that transcription of the GS- and GOGAT-encoding genes (*glnA* and *gltBD*, respectively) was induced 5- and 7-fold, respectively, under the aforementioned nitrogen conditions. When carbon was limiting (0.05% glucose), however, GS activity was reduced approximately 3-fold, and GOGAT activity was not induced under nitrogen starvation. Further studies with snake venom phosphodiesterase (SVPD) indicated the significance of adenylylation in the regulation of GS. Primer extension analysis revealed that transcription of the *glnA* gene starts at a G residue 109 base pairs upstream of the start of translation. The same transcriptional start site was employed irrespective of nitrogen or glucose status. Primer extension analysis of *gltBD* proved unsuccessful, presumably due to RNA secondary structure.

## 3.1 Introduction

When microorganisms are provided with an abundance of nutrients, they selectively use those that allow highest growth rates and those whose metabolism is most efficient in terms of energy requirements. Global regulatory pathways that allow the preferential assimilation of selected carbon and nitrogen sources have been identified in a host of organisms including bacteria, yeast and fungi (Fisher, 1999). Once these compounds have been assimilated, both the regulated and coordinated expression of the cellular pathways that metabolize them is crucial to the maintenance and ultimate survival of the organism. Nitrogen metabolism is no exception, and to this end, bacteria have evolved regulons and operons to execute this finely tuned regulation (Merrick and Edwards, 1995).

Most biochemical conversions are mediated by a single metabolic pathway. However, when seemingly redundant pathways exist that appear to duplicate another, detailed studies usually reveal that these parallel pathways are active under different conditions and often subject to different regulation (Helling, 1998). With regards to nitrogen metabolism, the classical example is that of the GS/GOGAT and GDH pathways of *E. coli* that both result in the production of glutamate. The GDH pathway seems to be employed during energy (carbon)-limited conditions, while the GS/GOGAT pathway is active when energy is sufficient (Helling, 1994). This phenomenon is the direct result of the energy requirements of the two pathways, as discussed later in this chapter.

In enterobacteriaceae, the NtrB and NtrC proteins form a two-component regulatory system that monitors and responds to intracellular nitrogen pools (Ninfa and Magasanik, 1986). Although no analogous two-component system has been identified in *C. glutamicum*, cellular GS activity is tightly regulated at both the transcriptional as well as posttranslational level (Schulz, 1996). The control of GS activity by adenylylation is the first line of regulation in response to sudden changes in nitrogen status (Stadtman, 1990). Adenylylation involves the addition of an AMP moiety to a conserved tyrosine residue in the GS protein. Removal of the AMP groups leads to the activation of enzyme activity, and

this adenylylation/deadenylylation process is pivotal in allowing rapid modification of cellular GS activity. The conserved tyrosine residue in the *C. glutamicum* GS protein is found within the peptide motif R-I-(X)9-L-Y (Schulz, 1996). This peptide sequence constitutes one of the signature motifs specific to GSI-β enzymes (Brown *et al.*, 1994). Snake venom phosphodiesterase (SVPD) has been used in this and several other studies to determine whether a GS enzyme is regulated by adenylylation (Patterson and Hespell, 1985; Bhatnagar *et al.*, 1986; Jakoby *et al.*, 1999). This particular type of phosphodiesterase catalyzes the removal of AMP groups from proteins, which in the case of GS results in increased enzyme activity.

Several enzymatic procedures have been developed to assay GS activity in bacterial systems (Stadtman *et al.*, 1979). These methods are based on the ability of the GS enzyme to catalyze a number of different reactions. The method employed in the research described here employs the γ-glutamyl transferase activity of GS (Shapiro and Stadtman, 1970). In contrast to GS activity, assaying for GOGAT activity directly employs the normal cellular reaction that is catalysed by the enzyme. Glutamine, α-ketoglutarate and NADPH are added to the CFE, and the rate of oxidation of the cofactor is monitored (Meers *et al.*, 1970). As NADPH is converted to $NADP^+$ by the activity of GOGAT, the optical density at 340nm decreases.

A vast number of studies have shown that the nitrogen assimilatory enzymes and many proteins involved in the metabolism of nitrogen in bacteria are regulated in response to nitrogen availability (reviewed by Merrick and Edwards, 1995 and Schreier, 1993). However, many of these enzymes are also subject to regulation by other molecules and cellular conditions, including carbon source and concentration (Tesch *et al.*, 1999). Ertan (1992b) did the first studies on how carbon sources affect the GS, GOGAT and GDH enzymes of coryneform bacteria. Of the carbon sources tested, he found that glucose and glutamate resulted in the highest GS and GOGAT activities in *C. callunae*. A more recent study on the effect of carbon on amino acid metabolism was performed by Parche and coworkers (2001) who investigated the phosphotransferase system (PTS) in *C. glutamicum*.

The findings of this study are discussed in section 3.4. In essence, both the availability and quality of energy and nitrogen source are the major factors that determine the participation of GS, GOGAT and GDH in nitrogen metabolism (Reitzer, 1998).

This chapter focuses on the contribution and regulation of GS and GOGAT in ammonia assimilation in *C. glutamicum* in response to nitrogen and carbon/energy status. These investigations were performed at both the RNA and protein level. The research described in this chapter was recently published (Schulz *et al.*, 2001).

## 3.2 Methods

### 3.2.1 Bacteria and growth conditions

The bacterial strains and plasmids used in this study are listed in Appendix A. Unless indicated otherwise, bacteria were routinely grown in LM (see section 2.2.1) at 37°C (*E. coli*) or 30°C (*C. glutamicum*) with aeration. For nitrogen studies, *C. glutamicum* strains were grown in MM (Siewe *et al.*, 1998) with 2mM or 100mM $NH_4Cl$ as the nitrogen source. The MM contained 2% glucose as the carbon source, unless indicated otherwise. Antibiotics were added where appropriate.

### 3.2.2 Preparation of cell free extracts and RNA

To prepare CFEs or RNA, *C. glutamicum* strains were grown in LM to logarithmic phase, washed twice with distilled water, and inoculated into LM with or without 1% glucose or MM with 2mM or 100 mM $NH_4Cl$. Cells were harvested and processed after 3 h incubation in these media. For CFEs, cells were washed with 50mM Tris-HCl pH 7.6, and the pellet was resuspended in 1 ml of the same buffer. The suspension was sonicated (Virsonic; total processing time 6 min, 1 min sonication on highest power setting, 1 min cooling interval), and the cell debris was removed by centrifugation (14,000 rpm, 5 min, 4°C). Dilutions of CFEs were made with 50mM Tris-HCl pH 7.6. For RNA extractions, cells were processed according to the protocol of Beg (1994) for Gram-positive bacteria.



### 3.2.3 GS and GOGAT assays

GS assays were performed according to a modification of the colorimetric γ-glutamyl transferase method of Shapiro and Stadtman (1970). One unit of GS activity is defined as the amount of enzyme that catalyzes the formation of 1μmol of γ-glutamylhydroxamate per min. GOGAT activity was determined using a modified method of the one described by Meers *et al.* (1970). The NADPH-GOGAT activity was measured using a reaction containing 50mM Tris-HCl buffer pH. 7.6, 15 mM glutamine, 3mM α-ketoglutarate (potassium salt, pH 6.8), 0.05mM NADPH and crude extract. The NADH-GOGAT assay

mixture contained 50mM potassium phosphate buffer pH 6.8, 5mM glutamine, 3mM α-ketoglutarate (potassium salt, pH 6.8), 0.05mM NADH and crude extract. GOGAT reactions were initiated by the addition of glutamine to reaction mixtures. One unit of enzyme is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NADPH or NADH per min. GOGAT assays were performed at room temperature. Specific activities for both GS and GOGAT are expressed as units per mg protein in the cell free extract. The activities displayed in the results are the mean of three independent experiments that differed from each other by no more than 20%. Protein concentrations were determined by the dye-binding method (Bradford, 1976).

### 3.2.4 SVPD treatment

CFEs were diluted to 1mg/ml protein and dessicated SVPD (Sigma, EC 3.1.4.1) was added directly to a final concentration of 10 mg/ml. The reaction was incubated at 37°C for 3h, and GS assays were subsequently performed. The SVPD had a specific activity of ca. 0.03 μmol of ρ-nitrophenol produced per min per mg protein at 37°C with bis(ρ-nitrophenyl phosphate) as the substrate.

### 3.2.5 RNA hybridizations

Total RNA (5μg) was loaded onto a Slot Blot® apparatus (Hoefer Scientific Instruments) and vacuum-blotted to Hybond $N^+$ membrane (Roche Diagnostics). The RNA was then UV-fixed to the membrane and probed according to the DIG RNA detection system (Roche). DIG-labeled DNA probes used were: *C. glutamicum glnA* (~1kb *Stu*I-*Xba*I fragment of pGS-2/71) and *C. glutamicum gltB* (~0.4kb *Sal*I-*Bgl*II fragment of pGOGup). Densitometric values of hybridization signals were determined using a Macbeth TD-901 transmission densitometer. Results are the mean of at least two independent experiments where the relative densities did not differ by more than 20%.

### 3.2.6 Primer extension analysis

This was preformed according to a method obtained from Miroslav Pátek (Institute of Microbiology, Czech Republic, personal communication). The Cy5-labeled primers that

were used were 5'-GAACTCGAC GTTTTCATCC-3' (*glnA*) and 5'-CAGGCGTCATGTTCATGC-3' or 5'-GGGTCGACCGTGGATATC-3' (*gltBD*). RNA (100μg) was ethanol-precipitated and resuspended in 100 μl HP (40mM PIPES pH 6.4, 1mM EDTA pH 8.0, 400mM NaCl, 80% formamide). Fluorescently-labeled primer (2-5 pmol) was added, followed by a 10 min denaturation at 96°C. Hybridization was then preformed at 42–55°C overnight. Water (300μl) and ethanol (800μl) were added, followed by a 30 min incubation at –20°C. The mixture was then centrifuged at 4°C and washed with 70% ethanol. The pellet was resuspended in 20μl RTB (4μl AMV reverse transcriptase buffer from Promega, 1μl dNTPs at 10mM, 1μl RNazin at 40U/μl from Roche, 2μl actinomycin D at 1mg/ml) and incubated at 42°C for 2 min. AMV reverse transcriptase (20U) (Promega) was then added, followed by incubation at 42°C for 2h. The reaction was stopped by the addition of 1μl EDTA (at 0.5M) and 1μl RNase A (at 10 mg/ml), followed by incubation at 37°C for 30 min. TES buffer (150μl; 10mM Tris-HCl, 1mM EDTA, 100mM NaCl, pH 7.5) and 500μl ethanol were added, and the mixture was incubated at –20°C for 30 min. Centrifugation and a 70% ethanol wash followed. The pellet was then resuspended in 7μl TE pH 7.6 and 3μl tracking dye. After a 5 min denaturation step at 96°C, all 10μl of the extension product was loaded onto the ALF-Express automated sequencer, and run alongside the appropriate sequencing lanes. In order to maintain the fluorescent integrity of the labeled primer, all primer extension procedures were performed away from artificial light.

## 3.3 Results

### 3.3.1 GS and GOGAT activities

Our initial enzyme studies involved quantitating the response of GS and GOGAT to nitrogen and carbon status in *C. glutamicum*. The bacterium was therefore grown in MM containing 2mM or 100mM $NH_4Cl$ or in LM, with and without 1% glucose added. GOGAT and GS assays were performed on CFEs. GS activity was approximately 5-fold higher in nitrogen-limiting than in nitrogen-rich conditions, while the activity was induced approximately 4-fold in the presence of glucose in complete medium (Fig. 3.1). GOGAT activity, on the other hand, was 7-fold higher in nitrogen-limiting than in nitrogen-rich medium, and showed no significant induction in the presence of glucose in complete medium. The GOGAT activity represented in Fig. 3.1 and 3.2 is NADPH-specific. Under the growth conditions used in this study, NADH-specific activity was less than 5% of NADPH-specific activity (data not shown).



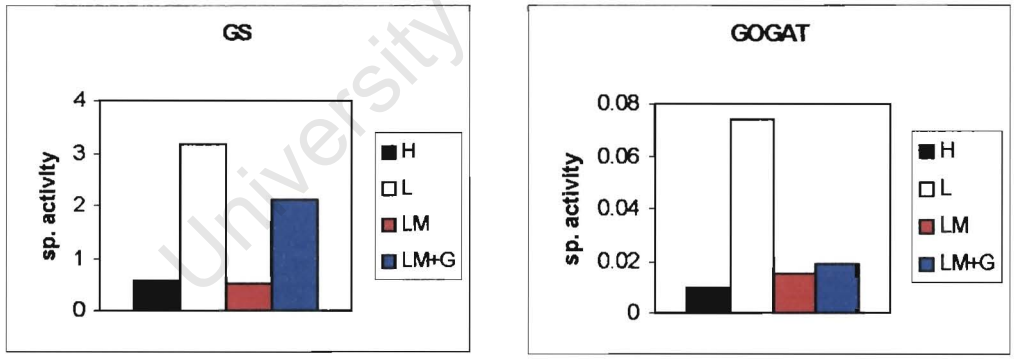



**Fig. 3.1** GS and GOGAT activities of *C. glutamicum* wild type grown in various media. High N: 100mM $NH_4Cl$ MM; Low N: 2mM $NH_4Cl$ MM; LM + G: LM with 1% glucose.

To further investigate the role of carbon concentration on the GS and GOGAT activity of *C. glutamicum*, cells were grown and processed as before, except that the glucose concentration of the MM was reduced 40-fold to 0.05%. As seen in Fig. 3.2, overall GS

activity is reduced by ca. 3-fold under carbon-limited conditions, although the relative induction of activity under nitrogen starvation is essentially maintained. In contrast, the induction of GOGAT activity under low nitrogen conditions was not seen when *C. glutamicum* was carbon limited.

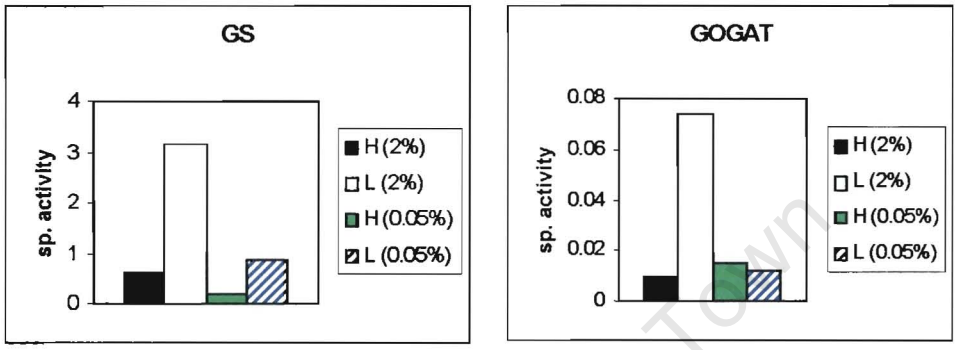



**Fig. 3.2** GS and GOGAT activities of *C. glutamicum* wild type grown under carbon excess (values taken from Fig. 3.1) and starvation. High N: 100mM $NH_4Cl$ MM; Low N: 2mM $NH_4Cl$ MM. Glucose concentrations are indicated in parentheses.

### 3.3.2 Posttranslational regulation of GS and GOGAT

Previous studies have shown that GS is also regulated by adenylylation in *C. glutamicum* (Jakoby *et al.*,1999). To determine what contribution posttranslational modification makes to induction of GS and GOGAT activity under low nitrogen, enzyme activities were measured in the absence of transcription and translation. *C. glutamicum* was grown in 100mM $NH_4Cl$ MM overnight, washed with distilled water, and resuspended in 2mM and 100mM $NH_4Cl$ MM with chloramphenicol and rifampicin (5μg/ml). The cultures were incubated for 20 minutes with shaking and cell extracts were processed as before. GS activity increased approximately two-fold, from 0.09 to 0.22 U $mg^{-1}$ when *C. glutamicum* was briefly exposed to nitrogen-limiting conditions in the presence of the antibiotics. GOGAT activity, however, was unaffected by the inducing conditions in the presence of antibiotics (0.004 U $mg^{-1}$ vs 0.005 U $mg^{-1}$). Posttranslational regulation of GS was confirmed by treating a CFE of *C. glutamicum* cells grown for 18h in nitrogen-rich MM

(100mM $NH_4Cl$, 2% glucose) with snake venom phosphodiesterase (SVPD) for 3 h prior to assaying for GS activity. The addition of SVPD resulted in a ca. 5-fold increase in GS activity from 0.30 U $mg^{-1}$ to 1.40 U $mg^{-1}$.

### 3.3.3 RNA hybridizations

To estimate transcriptional induction, total RNA was extracted from *C. glutamicum* grown under various conditions, and hybridized with *glnA*- and *gltB*-specific probes. Densitometer readings of RNA hybridization signals indicated that *glnA* and *gltB*(*D*) transcription are induced approximately 5- and 7-fold under low nitrogen conditions, respectively (Fig. 3.3). The presence of added glucose in complete medium also induced *glnA* transcription by around 10-fold, while *gltB*(*D*) transcription was essentially unaffected.

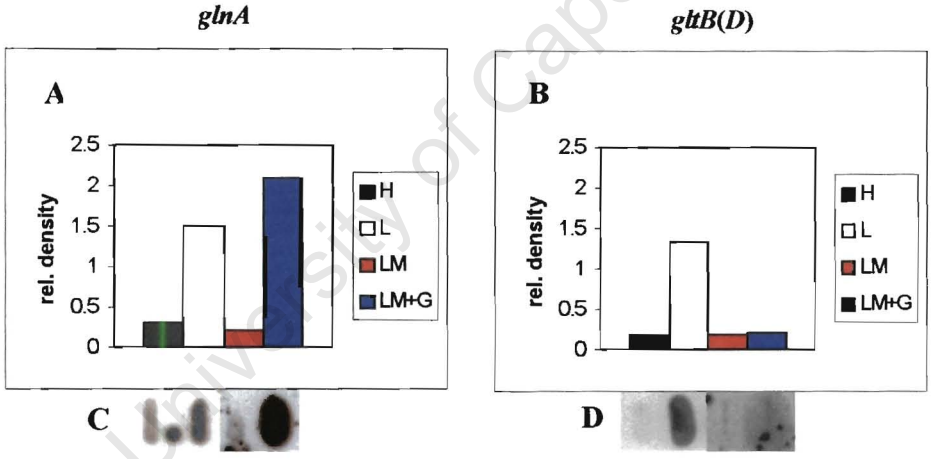



**Fig. 3.3 A & B**: Densitometric values of RNA hybridizations of *glnA* and *gltB*(*D*) transcripts from *C. glutamicum* wild type grown in various media. High N: 100mM $NH_4Cl$ MM, 2% glucose; Low N: 2mM $NH_4Cl$ MM, 2% glucose; LM + G: LM with 1% glucose added. Represented densities are relative to the background. **C & D**: Corresponding autoradiographs showing hybridization signals.

### 3.3.4 Primer extension analysis

Primer extension analysis was used to map the transcriptional start sites of the *C. glutamicum glnA* and *gltB(D)* genes under conditions of high and low $NH_4Cl$, and in complete medium in the presence or absence of glucose. In the case of *glnA,* it was found that the same transcriptional start site was employed irrespective of nitrogen concentration (Fig. 3.4 A & B). Similarly, it was found that the glucose status does not alter the start site of *glnA* transcription (Fig. 3.4 C). Transcription of this gene starts at a G residue 109 bp upstream of the start of translation (Fig 3.4 D). A region that is similar to the conserved -10 box found in many other bacteria was located at the corresponding position as the hexamer TAATAT. The –35 region comprised the sequence TTCAAA.

Significantly more primer extension product was detected in RNA isolated from cells subjected to nitrogen-limiting than nitrogen-rich conditions (see Fig. 3.4, B), confirming the 5-fold difference in RNA levels detected by hybridization (Fig. 3.3). Similarly, more product was detected when glucose was present (+g lane vs –g lane, Fig. 3.5 C). In the case of *gltB(D)* several attempts at primer extension were made, but no results were obtained. Up to 200μg of total RNA was used, and two different primers were designed. Neither primer nor protocol modification yielded extension products.

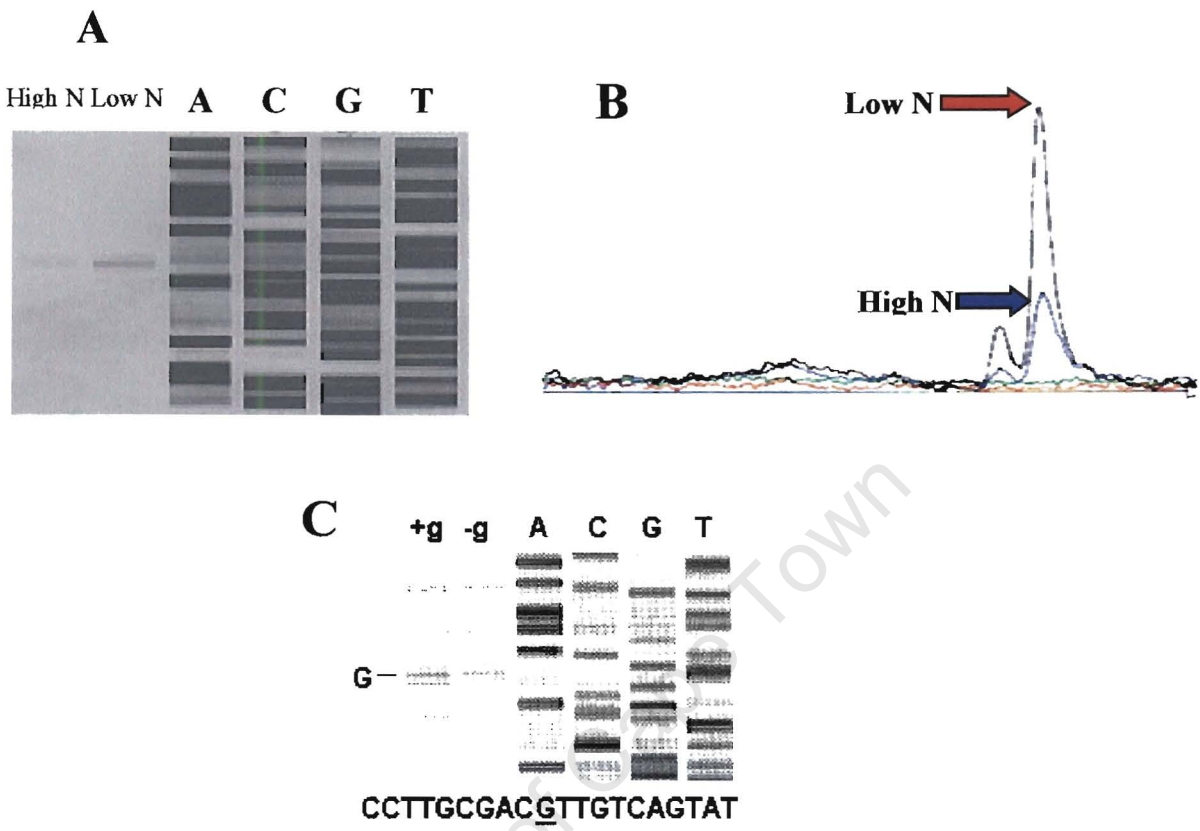





**Fig. 3.4** Primer extension analysis of *glnA* from *C. glutamicum* wild type grown in various media. **A and C:** Sequencing gels with primer extension products alongside the corresponding DNA sequences. High N: 100mM $NH_4Cl$ MM with 2% glucose; Low N: 2mM $NH_4Cl$ with 2% glucose; +g: LM + 1% glucose; -g: LM. **B.** Primer extension product peaks as interpreted by the ALF-Express software. **D.** Nucleotide sequence of the *glnA* promoter showing the –10 and –35 regions and the transcriptional start site (TS). The AmtR binding motif is underlined and indicated. The coding region of the *glnA* gene is indicated by the arrow and the start codon, CAC, is bolded.

## 3.4 Discussion

Various studies have been done on the effects of nitrogen source and concentration on the regulation of the nitrogen assimilatory genes of *C. glutamicum* (Nolden *et al.*, 2001; Jakoby *et al.*, 2000; Tesch *et al.*, 1999; Jakoby *et al.*, 1999; Beckers *et al.*, 2001). In our research, enzyme assays and RNA analyses revealed that GS and GOGAT activity and transcription of their encoding genes are enhanced under conditions of inorganic nitrogen starvation. GS and GOGAT activity were around 5- and 7-fold higher, respectively, when nitrogen was limiting. The GS activities in our studies are similar in magnitude to those obtained by Jakoby *et al.* (1999). At the transcriptional level, induction of *glnA* and *gltB(D)* in response to low nitrogen were also found to be 5- and 7-fold, respectively. In the case of GOGAT, our enzyme induction levels correlate well with those at the RNA level. This correlation seems consistent with the fact that the primary regulation of GOGAT activity is at the transcriptional level. In contrast, due to the posttranslational modification of GS, one would expect different induction levels of GS activity and *glnA* transcription in low nitrogen. Presently, the relative contribution that transcriptional and posttranslational regulation make to GS activity in *C. glutamicum* has yet to be precisely determined. Furthermore, we have found that it is often difficult to accurately quantify transcription by means of these Northern analyses, so the levels of induction described here should not be taken as absolute. The reasons for this are discussed in context in Chapter 4. Nonetheless, our RNA studies clearly show that transcription of both *glnA* and *gltBD* are significantly enhanced in *C. glutamicum* during nitrogen deprivation.

The experiments using chloramphenicol and rifampicin, potent inhibitors of transcription and translation, respectively, as well as SVPD, confirm posttranslational modification of GS. As expected, the GOGAT enzyme is not regulated by covalent modification. The studies using the antibiotics show that GS activity took approximately 20 min to double in the inducing (low nitrogen) medium. Jakoby and coworkers (1999) had similar results when they performed the reverse procedure i.e. starved the cells of nitrogen and then shocked them with 20mM $NH_4Cl$ for 30 minutes. It was found that GS activity decreased

by about 50% in the repressing condition. Thus it would appear that the rate of adenylylation (Jakoby's study) closely matches that of deadenylylation (this study). Jakoby goes on to suggest that this modest downregulation may be due to low ATase activity or the presence of a second GS enzyme. As far as our studies are concerned, slow deadenylylation rates were most likely due to the fact that the SVPD used was much lower in specific activity than those reported in other literature. Furthermore, pH discrepencies between the optima for GS activity and SVPD activity would also have resulted in a decreased SVPD reaction rate. However, this rather slow covalent modification rate is surprising, since flux analysis performed by Tesch *et al.* (1999) showed that the equilibration of ammonium across the *C. glutamicum* membrane took place within two minutes. One would therefore expect rapid covalent modification of the GS enzyme in response to $NH_4Cl$ concentration. Nonetheless, the substantial increase in *in vitro* activity (5-fold) found in our studies suggests that adenylylation of the GS enzyme plays a very significant role in the control of ammonia assimilation *in vivo*. While the results presented in this work corroborate Jakoby's suggestion that ATase activity may be low in *C. glutamicum*, other results discussed previously argue against the notion that a second, functional GS may be present in this bacterium. The most noteworthy of these is the fact that a *glnA* disruption mutant of *C. glutamicum* was a glutamine auxotroph (see Chapter 4).

To date, no instances of posttranlational modification of the GOGAT enzyme have been reported, although autocatalytic excision of a propeptide does occur (Stutz, 2000). Furthermore, and in contrast to GS, GOGAT has been shown to be insensitive to feedback inhibition by amino acids in *C. callunae* (Ertan, 1992a) as well as in *Bacillus licheniformis* (Schreier and Bernlohr, 1984) and *Rhodobacter capsulatis* E1F1 (Caballero *et al.*, 1989). The ca. 25% decrease in GS activity over the 3h incubation period at 37°C during the SVPD studies indicates that the GS enzyme is relatively stable under these conditions. In summary then, GS responds to nitrogen status at both the transcriptional and posttranslational level, while GOGAT seems to be regulated only at the transcriptional level.

The differences in complexity of regulation between GS and GOGAT are significant in terms of their metabolic functions. Firstly, the GOGAT enzyme is second in the metabolic sequence of ammonia assimilation, since GS provides GOGAT with its substrate, glutamine (Castaño *et al.*, 1992). It is therefore the GS enzyme that must be able to respond rapidly to changes in nitrogen conditions. Although it could be argued that GOGAT provides the glutamate required by GS, other enzymes including GDH, glutaminase, and transaminase are able to catalyse reactions that synthesize glutamate (Reitzer, 1998). GS, on the other hand, is the sole enzyme catalysing glutamine synthesis. Moreover, the GS-catalzed synthesis of glutamine from ammonia is considered to be a major branch point in cellular metabolism, since this amino acid functions as a nitrogen donor for the synthesis of carbamoyl phosphate, histidine, purines, pyrimidines, and glucosamine-6-phosphate, the precursor of peptidoglycan (Schreier, 1993).

GS and GOGAT activities were also responsive to carbon status. Under conditions of glucose limitation, GS activity was reduced ca. 3-fold. These findings are consistent with those of Helling (1998), who found that there was a sharp drop in GS activity in *E. coli* grown in glucose-limited medium. Under the carbon-limiting conditions studied here, GOGAT activity is not induced by low nitrogen, suggesting that the enzyme is functioning at a low basal level. Presumably, a longer growth time in the glucose-limited medium would result in a further drop in GOGAT activity. The reduced activities of these two enzymes during glucose-limitation can be explained in terms of the energy requirements for the GS/GOGAT pathway. Not only is ATP expended for every glutamate molecule that is formed (see reactions in introduction), but the adenylylation process regulating GS activity derives its AMP moiety from ATP (Reitzer, 1998). Furthermore, carbon shortage results in a decreased intracellular pool of α-ketoglutarate, which is a substrate for the GOGAT-dependent formation of glutamate. Although GDH has the same requirement for α-ketoglutarate, the reaction catalysed by this enzyme does not have the additional requirement for ATP. It is very likely, therefore, that GDH is favoured for the synthesis of glutamate formation during carbon/energy limitation. Indeed, *in vivo* flux experiments done by Tesch (1999) revealed that during chemostatic growth under carbon limitation and

ammonia excess, *C. glutamicum* assimilates only 28% of the $NH_4^+$ via the GS reaction as glutamine, while the remaining 78% was assimilated by GDH as glutamate. The importance of ATP in nitrogen assimilation is underscored by the finding that a *C. glutamicum* strain with a defective $H^+$-ATPase enzyme produces virtually no glutamate (Sekine *et al.*, 2001).

It is interesting to note that in complete medium, the addition of glucose results in a 4-fold increase in GS activity and a 10-fold increase in *glnA* transcription (Fig. 3.3). GOGAT activity and transcription, on the other hand, remain unaffected by glucose addition. While ATP requirements might explain how glucose concentration could affect the activity of the GS enzyme, some other type of regulation, possibly akin to catabolite repression, may be involved in transcriptional activation/derepression of *glnA* in *C. glutamium*. One possibility is that energy-rich conditions may cause cellular signals that result in the release of AmtR from the *glnA* promoter and derepression of transcription. However, the AmtR protein represses *glnA* as well as *gltBD* transcription (Nolden *et al.*, 2001), and we have shown here that excess glucose does not result in activation (derepression) of *gltBD* transcription. It has been suggested that the $P_{II}$ protein may interact with AmtR (Jakoby *et al.*, 2000), and since the activity of $P_{II}$ in other bacteria is affected by ATP and α-ketoglutarate binding (Atkinson and Ninfa, 1999), the same may hold true in *C. glutamicum*. If this is the case, then energy and nitrogen status signals could be relayed to AmtR via $P_{II}$. This hypothesis is revisited in Chapter 5.

In another Gram-positive bacterium, *B. subtilis*, ammonium assimilation is subject to regulation by the catabolite control protein, CcpA, as well as nitrogen regulators (Faires *et al.*, 1999). As mentioned in Chapter 2, when Schmid and colleagues (2000) examined the proteins that were synthesized during nitrogen starvation in *C. glutamicum*, they found that several of them were enzymes of central carbon metabolism. Furthermore, 2-D PAGE analysis and the measurement of oxygen consumption revealed an increase in energy demand during nitrogen starvation.

Parche *et al.* (2001) recently investigated the significance of the PTS in amino acid metabolism in *C. glutamicum.* The PTS is involved in carbohydrate uptake, carbon regulation and chemotaxis in low-GC Gram-positive and in Gram-negative bacteria (reviewed by Postma *et al.*, 1993). The existence of this system in *Corynebactericeae* was first reported by Mori and Shiio in 1987, and PTSs that are specific for glucose, sucrose, fructose and mannose have subsequently been identified in *C. glutamicum* (Parche *et al.*, 2001). Parche and coworkers have demonstrated that carbon repression of glutamate uptake is exerted by the PTS when *C. glutamicum* is grown in medium with glucose or fructose as carbon sources. The mechanism of this repression is, however, still under investigation. All the experimental evidence cited above clearly indicates that distinct signaling and regulatory pathways exist that couple carbon and nitrogen metabolism in *C. glutamicum*.

In terms of the coenzyme specificity for GOGAT, Ertan (1992) found that this enzyme possesses both an NADPH- and NADH-dependent activity in *C. callunae*. The NADH-dependent activity was, however, only 25% of the NADPH-activity, and was virtually absent when the cells were grown with $NH_4Cl$ as the nitrogen source. In the case of *C. glutamicum* and under the conditions studied here, GOGAT activity was similarly predominantly NADPH-dependent. For this reason, subsequent studies by other researchers have focused almost exclusively on the NADPH-specific activity of the *C. glutamicum* GOGAT.

Subsequent to our research, Nolden and coworkers (2001) confirmed that the start of transcription of the *C. glutamicum glnA* gene occurs at a G residue 109 bp upstream of the structural gene. The *C. glutamicum glnA* promoter region was found to contain a –10 consensus sequence (TAATAT), which correlates well with the findings of Pátek *et al.* (1996). They studied the promoter regions of 33 different genes in *C. glutamicum*, and concluded that the region 10 bp upstream of the transcriptional start site comprises the hexamer TAnAAT or a similar motif. Pátek found the –35 region to be less conserved, but also identified a motif, ttGcca. The –35 region of the *C. glutamicum* 13032 *glnA* promoter (TTCAAA) only partially conforms to this motif. The finding that more primer extension

product was produced under low nitrogen conditions and in the presence of glucose indicates increased levels of the corresponding RNA molecules and thus reinforces the results obtained from the RNA hybridization studies. Furthermore, our results show that the same transcriptional start site is employed irrespective of glucose or nitrogen status. This is in contrast to other bacterial systems, such as that of *E.* coli, where the *glnA* gene is under the control of two different promoters, depending on nutrient status (Woods and Reid, 1993). As mentioned previously, Nolden and colleagues (2001) identified and experimentally confirmed an AmtR binding motif upstream of the *C. glutamicum glnA*. This sequence comprises the bases TATCAT and is situated at positions –27 to –32 relative to the start of transcription. Binding of the AmtR protein to this region obviously excludes binding of the RNA polymerase which in turn prevents transcription of *glnA*.

Primer extensions of *gltB*(*D*) were performed using up to 200 µg of RNA, which is twice the amount prescribed by the protocol. It was thought that since the *gltB*(*D*) was likely to be cotranscribed as one large transcript it would represent only a small proportion of the transcripts in the total RNA and would thus not be sufficiently detectable. Increasing the total amount of RNA used for the extension might have overcome this problem. This was not the case, however. With regards to using two different primers, the distance between the primer and the transcriptional start site is an important factor influencing primer extension analysis. Firstly, the reverse transcriptase enzyme only has limited processivity, and, secondly, the automated sequencer is only able to detect DNA fragments in excess of around 50 bp in length. The two different primers used in our studies were 27 and 64 bp downstream of the translational start site. Presumably, secondary structure formation of the RNA under these conditions prevented either the binding of the labeled primer to the RNA or the formation of a detectable extension product due to reverse transcriptase obstruction. Subsequent to our studies, Beckers and colleagues (2001) established that the *gltB* and *gltD* genes of *C. glutamicum* are indeed cotranscribed, and they employed the 5'-3' RACE kit (Roche Diagnostics, Germany) to determine that the transcriptional start site occurs at a C residue 41 bp upstream of the *gltB* structural gene. This kit employs a reverse transcriptase PCR technique, and the cyclic denaturation steps prevent mRNA secondary structure

formation. Beckers showed that a –10 consensus motif typical of *C. glutamicum* promoters (Pátek *et al.*, 1996) was present as TATTAT, although this region occurred at positions –1 to –6 relative to the start of transcription. The –35 consensus motif was located at the appropriate position as the nucleotide sequence TTGCGT. The binding site for the AmtR repressor protein is located upstream of the –35 consensus sequence at positions –109 to –102 relative to the start of *gltBD* transcription. The genetic organization of the region upstream of *gltB* in *C. glutamicum* is dealt with in more depth in the following chapter.

Having established the pivotal role of GS in nitrogen assimilation in *C. glutamicum*, we wanted to investigate what effect disruption of *glnA* would have on nitrogen metabolism. Also, closer analysis of the *gltBD* region identified an interesting gene directly upstream of the operon, and the effect of disruption of this gene was studied in parallel with the *C. glutamicum glnA* mutant.

# Chapter 4

## Elucidation of the Roles of *glnA* and *hkm* in Nitrogen Assimilation in *C. glutamicum*


### Abstract

Sequence analysis identified a putative gene directly upstream of *gltB* in *C. glutamicum*, which we designated *hkm*. This gene may encode a putative sensor protein that contains a conserved PAS domain. Gene inactivation techniques were used to generate *glnA* and *hkm* disruption mutants of *C. glutamicum*, designated GLNA19-1 and HKM19, respectively. Southern hybridization analyses confirmed integration of the respective replacement vectors into the chromosomes of the mutants. Growth of the mutants on MM with various nitrogen sources revealed that GLNA19-1 had an absolute requirement for glutamine, while HKM19 was able to grow on all nitrogen sources tested. GLNA19-1 displayed no detectable GS activity, while the nitrogen-dependent GS activity profile of HKM19 was the same as that of wild type *C. glutamicum*. GOGAT activity, on the other hand, was unaffected by *glnA* disruption. In contrast, repression and induction of GOGAT activity in response to 100mM $NH_4Cl$ and 2mM $NH_4Cl$, respectively, was reduced around two-fold in HKM19. These results were confirmed by RNA hybridization studies which revealed reduced *gltBD* transcription in HKM19. Promoter fusion experiments showed that transcription of the *hkm* gene occurred at a low level, and was not significantly affected by nitrogen concentration. Our studies suggest that the *hkm* gene may encode a signaling protein involved in the control of GOGAT activity in *C. glutamicum*.

## 4.1. Introduction

One of the most efficient methods of determining the importance and cellular function of a gene is by inactivating it and studying the effect. Consequently, gene disruption and replacement techniques have been extensively used for genome mapping, insertional mutagenesis, gene cloning, recombinant DNA stabilization and the study of gene function and expression (Vertès *et al.*, 1993; Jakoby *et al.*, 2000; Fink *et al.*, 1999; Pátek *et al.*, 1994; Schwarzer and Pühler, 1991; Reyes *et al.*, 1991). An obvious advantage of this technique over transposon or chemical mutagenesis is that specific genes can be targeted for inactivation. A major obstacle, however, is that the genomes of some organisms are not easily accessible to exogenous DNA due to thick cell walls and restriction systems (Schäfer *et al.*, 1990). Fortunately, *C. glutamicum* can be both electroporated (Bonamy *et al.*, 1990) and conjugated (Schäfer *et al.*, 1990) at high efficiencies. Conjugation mediated by the broad host range plasmid RP4 has been used extensively to transfer plasmid DNA from *E. coli* to a wide range of Gram-negative bacteria (Schäfer *et al.*, 1994). RP4 is also able to mobilise plasmids from *E. coli* at relatively high efficiencies to several Gram-positive genera including *Corynebacterium*, *Streptococcus*, *Mycobacterium*, *Bacillus*, *Arthrobacter*, and *Streptomyces*.

To facilitate the genetic manipulation of coryneform bacteria, Schäfer and colleagues (1994) combined the transfer machinery of RP4 with two suitable cloning vectors pK18 and pK19. These plasmids are derivatives of pBR322, which means their host range is restricted to *E. coli* and closely related species (Bolivar *et al.*, 1977). These researchers further equipped these mobilizable derivatives, designated pK18*mob* and pK19*mob*, with a modified *Bacillus sacB* gene whose expression confers sucrose sensitivity to Gram-negative and some Gram-positive bacteria including *C. glutamicum* (Jäger *et al.*, 1992). The resultant vectors were pK18*mobsacB* and pK19*mobsacB* (Fig. 4.1). These plasmids are similar to those employed in Gram-negative allelic replacement protocols (Selbitschka *et al.*, 1993), but provide a combination of several advantages: 1) the vectors are small which means large fragments can be cloned into them, 2) they carry the Tn*5* Km resistance gene which is a highly efficient selection marker in bacteria (Parke, 1990), 3) the cloned inserts can be directly sequenced using the Bluescript SK

M13 sequencing primers, 4) plasmids with inserts cloned into the multiple cloning site can be identified by *lac* blue-white selection, and 5) a multiple cloning site with several unique restriction enzyme sites allows convenient cloning (Schäfer *et al.*, 1994).

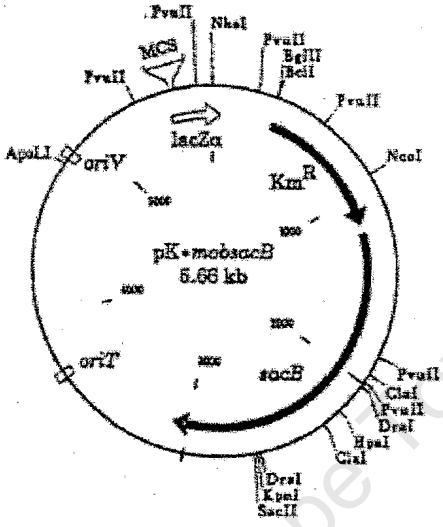



**Fig. 4.1** Physical map of the mobilizable replacement vector pK18*mobsacB*/pK19*mobsacB* (Schäfer *et al.*, 1994.)

The desired recombinant vector constructs can then be transferred to *E. coli* to *C. glutamicum* by conjugation. Since the vector is unable to replicate in *C. glutamicum*, the only way that Km resistance can be conferred to *C. glutamicum* is by integration of the vector into the chromosome via homologous recombination. Disruption mutants display Km-resistance and sucrose-sensitivity. Excision of the plasmid can then selected for on a medium containing 10% sucrose. Cells that are able to grow on this medium have lost the plasmid due to a second cross-over event that either restored the wild type, or leads to generation of the mutant strain with the disrupted sequence. Essentially, the *sacB* gene allows the selection of rare double crossover mutants.

Two *C. glutamicum* genes were selected for inactivation in the studies described here, namely *glnA* and *hkm*. Disruption of *glnA* would reveal whether *C. glutamicum* has a second functional GS enzyme that is able to produce glutamine. The second gene, named *hkm* (histidine kinase from *Mesorhizobium*), which occurs directly upstream of *gltBD* operon, has high homology to a sensor histidine kinase from *Mesorhizobium loti*.

In another Gram-positive organism, *B. subtilis*, the *glt* genes are under the control of the GltC protein whose encoding gene occurs upstream of, and in the opposite direction to the *glt* genes (Bohannon *et al.*, 1985)(Fig. 4.2). The promoter regions of *gltC* and *gltAB* overlap, and the presence of several inverted repeats is thought to play a role in regulating transcription. Transcription of *gltC* does not appear to be subject to nitrogen regulation, and transposon mutagenesis studies have shown that GltC is a negative regulator of its encoding gene (Schreier, 1992). The primary sequence of GltC contains a helix-turn-helix region that is similar to those found in DNA binding proteins. Deletion of this motif results in glutamate auxotrophy, confirming its functional requirement. During growth of *B. subtilis* in glutamate-limited medium, GltC activates transcription of *gltAB*. Like *gltC*, the *C. glutamicum hkm* gene also occurs directly upstream of the GOGAT-encoding genes and is also transcribed in the opposite direction. Our aim was therefore to disrupt the *hkm* gene, and establish whether its product might be involved in *gltBD* regulation in *C. glutamicum*.

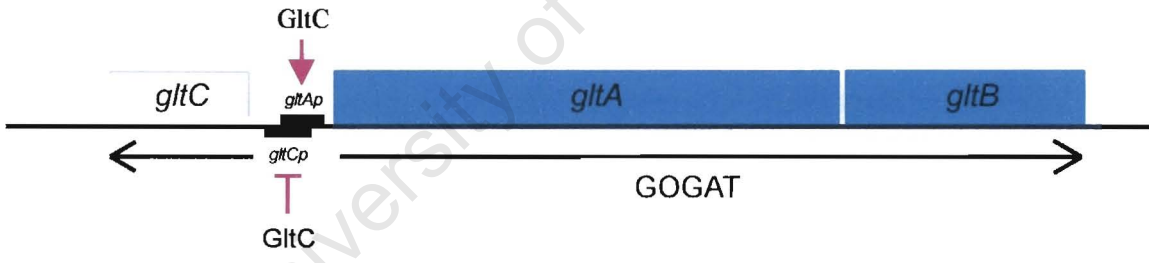



**Fig. 4.2** Regulation of *gltAB* transcription in *B. subtilis*. The product of the *gltC* gene, GltC, represses it's own synthesis and is required for the activation of *gltAB*. The two promoters (*gltAp* and *gltCp*) overlap. See text for explanation. Adapted from Schreier (1992).

This chapter describes the generation and characterization of *C. glutamicum* disruption mutants of *glnA* and *hkm*. Growth studies on various nitrogen sources, enzyme assays, promoter fusion experiments, and RNA hybridizations were employed in the course of these investigations.

## 4.2 Methods

### 4.2.1 Bacteria, plasmids and media

The bacterial strains and plasmids used in this study are listed in Appendix A. Unless indicated otherwise, bacteria were routinely grown in LM (see section 2.2.1) supplemented with the appropriate antibiotics. *E. coli* and *C. glutamicum* strains were grown aerobically at 37°C and 30°C, respectively. The MM (Siewe *et al.*, 1998) contained various nitrogen sources at the indicated concentrations.

### 4.2.2 Preparation and manipulation of DNA

As in section 2.2.2.

### 4.2.3 Construction of *C. glutamicum glnA* disruption mutants

*4.2.3.1 Construction of integration vector*

A ~660 bp *BamH*I-*Sph*I fragment internal to the *glnA* gene of *C. glutamicum* ATCC 13032 was isolated from pGS-2/71. This fragment was ligated with *BamH*I-*Sph*I-digested pK19*mobsacB*. The ligation mixture was transformed into *E. coli* JM109 (Sambrook *et al.*, 1989). A selected, confirmed recombinant plasmid, designated pK19*glnA*, was transformed into *E.coli* S17-1 by electroporation (Ausubel *et al*, 2001).

*4.2.3.2 Mating experiments*

The mating protocol was scaled up and is a modification of the method used by Schäfer *et al.* (1990). The *E. coli* S17-1 donor strain, harbouring pK19*glnA*, was grown to late exponential phase at 30°C in 100 ml YT medium (Sambrook *et al.*, 1989) supplemented with Km. The recipient *C. glutamicum* ATCC 13032 wild-type strain was grown to stationary phase in 100 ml YT at 30°C, followed by heating in a 200ml GSA centrifuge tube at 48.5°C for 30 min. The two cultures were then mixed (donor:recipient ratio of 1:1, approximately), after which the cells were harvested by centrifugation (5,000 rpm, 5 min, 20°C). The mating mixture was then resuspended in 1 ml YT (Sambrook *et* al., 1989) and spread onto YT agar (Appendix B) plates. Plates were incubated for 20 h at 30°C, and cells were subsequently washed from the plates in 1 ml YT. The YT cell suspension was then plated onto YT agar supplemented with 50µg/ml Nx and Km,

followed by incubation at 30°C for 3 days. *C. glutamicum glnA* mutants were generated by Helen Collett, Univ. of Cape Town, South Africa.

### 4.2.4 Isolation and cloning of region upstream of *C. glutamicum gltB*

PCR was used to isolate a ca. 1.05kb fragment comprising a small region of the N-terminal sequence of *gltB* and ca. 550bp upstream of this gene (most of which is *hkm* coding sequence). Amplifications were performed using the GC-rich PCR Kit (Roche Diagnostics). Reaction mixtures (50 µl) contained *C. glutamicum* genomic template DNA (100 ng), primers (0.5 µM each), dNTPs (50 µM each), $MgCl_2$ (2.0 mM), Taq polymerase (2U) and its buffer. After an initial denaturation step (93°C, 2 min), the samples were subjected to 30 amplification cycles (30s denaturation at 93°C, 30s annealing at 53°C, 60s elongation at 72°C) using a GeneAmp PCR System 9700 (Applied Biosystems). The following primers were used: Forward: 5' AAT GCT GCC ACG CCT TCG 3', Reverse: 5' CGC TCA CAA CGC TTA CGG 3'. Primers were synthesized by Pei-Yin Ma (Dept. of Molecular and Cell Biology, Univ. of Cape Town, South Africa) using a Beckman Oligo 1000M DNA Synthesizer. The PCR product was cloned into pGEM using the Rapid Ligation Kit (Promega). The resultant recombinant plasmid was confirmed by sequencing and designated pGOGup.



### 4.2.5 Construction of *C. glutamicum hkm* disruption mutant

*4.2.5.1 Construction of integration vector*

A ~400 bp *Xmn*I-*Sph*I fragment comprising most of the *C. glutamicum* ATCC 13032 *hkm* gene was isolated from pGOGup and ligated with *Pst*I-digested, blunted pK19*mobsacB*. The ligation mixture was transformed into *E. coli* JM109 (Sambrook *et al.*, 1989). A selected and confirmed recombinant plasmid, designated pK19*hkm*, was subsequently transformed into *E. coli* S17-1 by electroporation (Ausubel *et al.*, 2001).

*4.2.5.2 Mating experiments*

This protocol was obtained from Lyndon Oldfield (AECI, South Africa). The *C. glutamicum* ATCC 13032 recipient strain was grown O/N in buffered LM (20g/L tryptone, 20g/L yeast extract, 5g/L NaCl, 8g/L $K_2HPO_4$, 2g/L $KH_2PO_4$, 15g/L glucose) with 50µg/ml Nx to an $OD_{600}$ of 5.0-6.0 (8 cultures were prepared). An O/N culture of the *E. coli* S17-1 donor strain harbouring pK19*hkm* was prepared in LM which was then

inoculated into 50ml (1:50 dilution) LM. The donor was grown to an $OD_{600}$ of approximately 1.0. *C. glutamicum* cultures were dispensed (10ml volumes) into glass standard containers and heated at 48.5°C for 9 min. Subsequently, 1ml of the donor and 3ml of each of the 8 heat-treated recipient cultures were mixed and harvested by centrifugation (6000rpm, 5min, RT). The cell pellets were then resuspended in 200µl LM and pipetted onto a 0.45µm cellulose acetate filters (Millipore Cat. No. HAWP 02500)(1 filter/pellet) which were placed onto LM plates. Incubation at 30°C for 20h followed. Cells were then washed off the filters in 1ml LM and dilutions were plated onto brain heart infusion agar plates supplemented with 50µg/ml Nx and Km. Plates were incubated at 30°C for 2-3 days.

### 4.2.6 Southern hybridizations

Restriction enzyme digests of the appropriate DNA were separated in a 0.8% agarose gel in Tris-acetate buffer. Southern blotting was performed as described by Sambrook *et al.* (1989) using Hybond-$N^+$ nylon membranes (Amersham). Hybridization (at 68°C), washing and detection procedures were performed according to the DIG detection (Roche Diagnostics). DIG-labeled probes used were: ~1.0kb *Stu*I-*Xba*I fragment of pGS-2/71 (*glnA*-specific), ~400bp *Sal*I-*Bgl*II fragment of pGOGup (*gltBD*-specific), and ~240bp *Eco*RI-*Xmn*I fragment of pGOGup (*hkm*-specific).

### 4.2.7 Growth profiles

O/N cultures of *C. glutamicum* strains were grown in 10 ml LB (Sambrook *et al.*, 1989) with Nx (and Km, where appropriate) with aerated incubation at 30°C. The O/N cultures (1ml volumes) were microfuged for 5 min to harvest the cells, and pellets were washed twice with distilled water. Each 1ml pellet was then inoculated into 50 ml MM containing the selected nitrogen source(s) and 50µg/ml Nx or Km (where appropriate). Flasks were incubated at 30°C with aeration. The optical density (OD) at 600nm of all cultures was determined at selected time intervals. Growth studies were performed in duplicate and the mean values of the two experiments were used to plot growth profiles.

### 4.2.8 GS and GOGAT assays

CFEs were prepared from *C. glutamicum* strains as described in section 3.2.2. GS and GOGAT assays were performed as described in section 3.2.3.

### 4.2.9 RNA hybridizations

RNA was prepared as described in section 3.2.2. Total RNA (15µg) was loaded onto a Slot Blot® apparatus (Hoefer Scientific Instruments) and was vacuum-blotted to Hybond $N^+$ membrane (Roche Diagnostics). The RNA was then UV-fixed to the membrane and probed according to the DIG RNA detection system (Roche Diagnostics). The *gltBD*-specific DIG-labeled DNA probe used was the ~0.4kb *Sal*I-*Bgl*II fragment of pGOGup. Densitometric values of hybridization signals were determined using a Macbeth TD-901 transmission densitometer. Results are the mean of at least two independent experiments where the relative densities did not differ by more than 20%.

### 4.2.10 Promoter *cat* fusion studies

A ~570bp *EcoR*I-*EcoR*V fragment, encompassing ca. 330 bp of coding sequence and ca. 240bp upstream of the *C. glutamicum hkm* gene, was isolated from pGOGup, filled-in with T4 polymerase, and cloned into *Sal*I-digested pEKplCm. The ligation mixture was subsequently transformed into *E. coli* DH5α*mcr* (Sambrook *et al.*, 1989). A selected transformant (containing a recombinant plasmid whose insert identity and orientation was confirmed by sequencing) was grown up for a Nucleobond® plasmid extraction. The plasmid DNA was introduced into *C. glutamicum* ATCC 13032 by electroporation (Appendix B) followed by selection on LA containing 50 µg/ml Km and 5 µg/ml Cm. CAT assays of *C. glutamicum* containing the promoter fusion were performed as described in section 2.2.9.

## 4.3 Results

### 4.3.1 Sequence analysis of *gltBD* upstream region

We have previously shown that no sequences involved in nitrogen regulation or metabolism flank the *glnA* gene in *C. glutamicum* (Schulz, 1996). The regions up- and downstream of *gltBD*, however, have yet to be investigated. The *gltBD* region of *C. glutamicum* ATCC 13032 was initially sequenced by Kanno *et al.* in 1999 (GenBank Accession No. AB024708).


**Fig. 4.3** Genetic organisation of the region upstream of the *C. glutamicum gltBD* operon. Coding regions of *hkm* and *gltB* are blue and underlined. Black arrows indicate gene orientation. The AmtR binding site (BS), transcriptional start (ts in red) site of *gltBD*, and –10 and –35 consensus regions are shown. The stop codon is indicated with an asterisk. Possible –10 and –35 regions for *hkm* are shown in parentheses. The sequence between the red arrows was used to construct the *hkm* replacement vector. Bases are numbered according to the original DNA contig (GenBank Accession No. AX120085).

The sequence lodged in the database by these researchers comprised the complete coding sequences of the *gltB* and *gltD* genes as well as approximately 560bp upstream and 2120bp downstream. Sequence analysis using ORF Finder (www.ncbi.nih.nlm.gov) revealed the existence of a partial open reading frame (ORF) directly upstream of, and in the opposite orientation as, *gltB*. Interestingly, a gene encoding a transposase enzyme occurs upstream of this partial ORF. Downstream of *gltD*, homology searches identified an ORF encoding a putative protein of 495 amino acids with very high homology (E value = $10^{-117}$) to the *M. tuberculosis* hypothetical protein Rv3698. Subsequent completion of sequencing the entire *C. glutamicum* ATCC 13032 genome sequence (2001) allowed the determination of the complete sequence of the partial ORF upstream of *gltB* (GenBank Accession No. AX120085)(Fig. 4.3). Further investigation revealed that the putative protein encoded by this ORF had high homology (E value = 6 x $10^{-14}$) to a histidine kinase of a two-component regulatory system from the nitrogen-fixing bacterium *Mesorhizobium loti*. The gene was therefore named *hkm*.

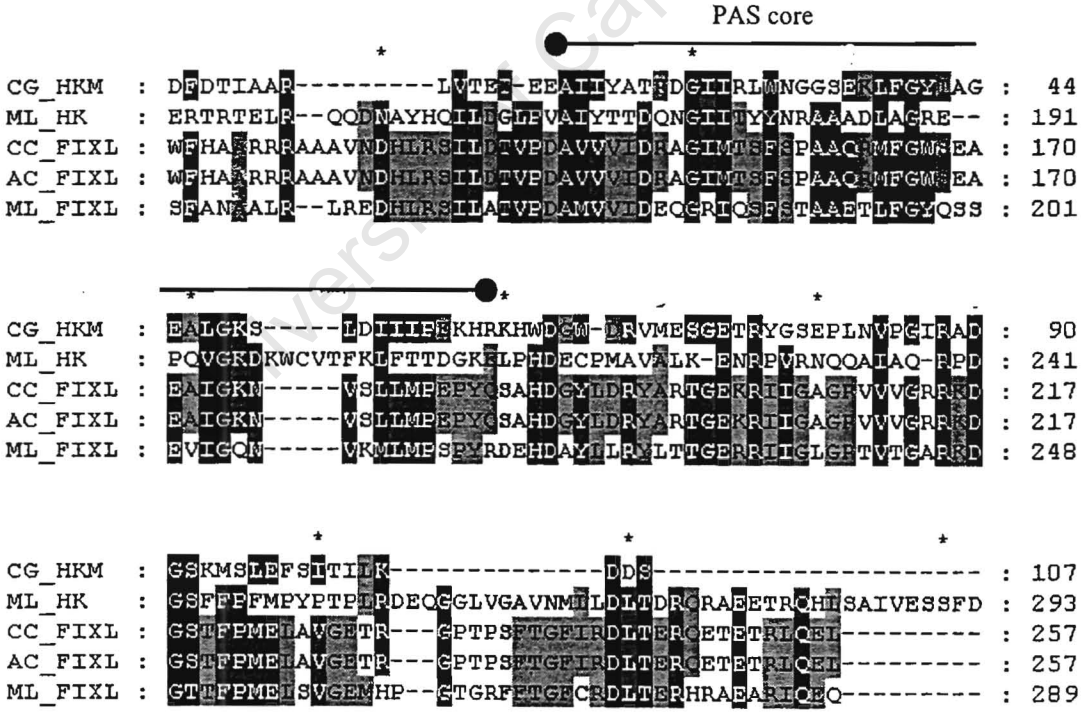



**Fig. 4.4** Multiple sequence alignment of the deduced amino acid sequences of *C. glutamicum* Hkm (CG_HKM) and the N-terminal regions of several signal transducing proteins. ML_HK: *M. loti* histidine kinase of two-component regulatory system (GenBank Acc. No. NP_109506.1); CC_FIXL: *Caulobacter crescentus* FixL sensor protein (GenBank Acc. No. AAK22744.1); AC_FIXL: *Azorhizobium caulinodans* FixL sensor protein (GenBank Acc. No. P26489); ML_FIXL: *M. loti* FixL sensor protein (GenBank Acc. No. NP_107078.1). The conserved PAS core domain is indicated (see text). Amino acids are numbered.

Possible –10 and –35 consensus motifs for the promoter of *hkm* are shown in Fig. 4.3. The complementary sequences of these regions conform to the consensus motifs proposed for *C. glutamicum* promoters by Pátek *et al.* (1996). The *hkm* gene is transcribed in the opposite direction as *gltBD* and encodes a putative protein, Hkm, which is 145 amino acids in length. An intergenic region of 134bp separates the coding regions of *hkm* and the gene encoding the transposase. No inverted repeats with the potential to form stable stem-loop structures could be identified downstream of *hkm*. Multiple sequence alignment indicated that Hkm shares sequence similarity to the N-terminal region of other histidine kinases, including some involved in nitrogen regulation (Fig. 4.4).

The conserved domain search (CD-Search, www.ncbi.nih.nlm.gov) showed that Hkm contains a PAS core domain (E value = $2 \times 10^{-5}$)(Fig. 4.4). This motif consist of around 40 to 60 amino acids (usually glycine-rich) with several conserved residues, and has been identified in proteins from all three kingdoms, *Archae*, *Bacteria*, and *Eucarya* (Zhulin *et al.*, 1997). PAS is an acronym derived from the names of three proteins in which this motif was recognized: the *Drosophila* period clock protein (PER), vertebrate aryl hydrocarbon receptor nuclear translocator (ARNT), and *Drosophila* single-minded protein (SIM) (Taylor and Zhulin, 1999). The complete PAS domain is a region of around 100 amino acids and consists of the conserved core (Conserved Domain: Pfam00989, www.ncbi.nih.nlm.gov) and a β-scaffold that are joined by a helical connector. Secondary structure analysis indicated that Hkm does not conform to the typical PAS domain protein structure (Fig. 4.5). Although a random coil region connects the PAS core to the C-terminal region of the protein, no predominance of β-sheets occurs in the C-terminus. In fact, the C-terminal region is rich in amino acids that are associated with α-helices. In current literature, the term "PAS domain" usually refers to the PAS core, while in some cases it denotes the entire PAS region. In our discussions, all subsequent referrals to a PAS domain will denote the PAS core.

The three eukaryotic proteins mentioned above all have two PAS domains that may have divergent origins (Taylor and Zhulin, 1999). In contrast, PAS domains in prokaryotic proteins are often present as single copies. These domains are signaling

modules that monitor changes in light, redox potential, oxygen, small ligands and the overall energy levels of the cell. The PAS module has been identified in a variety of sensor proteins including histidine and threonine/serine kinases, chemoreceptors and photoreceptors for taxis and tropism, circadian clock proteins, voltage-activated ion channels, and cyclic nucleotide phosphodiesterases.

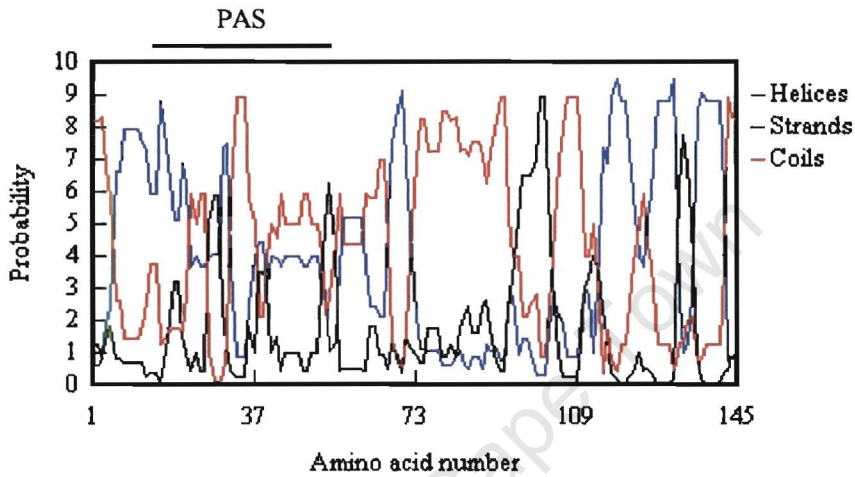



**Fig. 4.5** Predicted secondary structure profile of the putative Hkm protein. Helices: α-helices; Coils: random coils; Strands: β-sheets. The PAS domain is shown.

PAS domains are found in combination with a variety of regulatory motifs in multi-domain proteins, including some involved in nitrogen metabolism such as FixL, NtrB and NifU (Zhulin *et al.*, 1997). The role of PAS motifs in various sensory proteins is reviewed in greater detail in the discussion of this chapter.

The multiple sequence alignment indicated the putative Hkm protein is much smaller in comparison to known histidine kinases which are often in excess of 500 amino acids. Consequently, other domains that are normally present in histidine kinases, such as the phosphoacceptor (H-box), regulator receiver, and ATPase domains, are absent in Hkm.

Both the genetic location of *hkm* and the occurrence of a PAS domain in the putative protein encoded by the gene prompted us to investigate whether *hkm* may be involved in GOGAT activity. Furthermore, since we had performed numerous studies on GS and *glnA* regulation in *C. glutamicum*, we wanted to study the effect of inactivation of the

*glnA* gene. We therefore generated *C. glutamicum glnA* and *hkm* disruption mutants and studied them in parallel.

### 4.3.2 Construction and confirmation of *glnA* disruption mutants

In order to construct a *glnA* replacement vector, a fragment internal to the *glnA* gene of *C. glutamicum* was cloned into plasmid pK19*mobsacB*. The resultant recombinant plasmid, designated pK19*glnA*, was subsequently electroporated into *E. coli* S17-1, and transferred to *C. glutamicum* by conjugal mating.

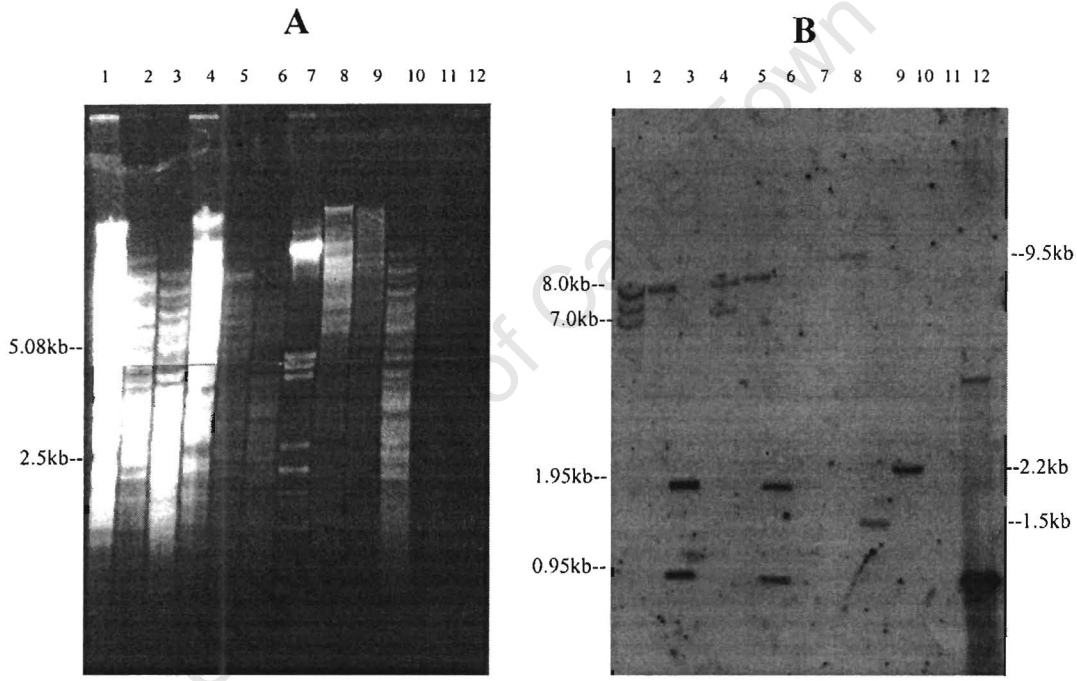



**Fig. 4.6**. Southern hybridization to confirm inactivation of the *C. glutamicum glnA* gene. **A**: Agarose gel electrophoresis of DNA restricted with indicated enzymes. Lane 7: λ-*Pst*I molecular markers; lane 12: 10ng pGS-2/71, *Stu*I-*Xba*I; lanes 1-3: Chromosomal DNA from putative *C. glutamicum glnA* mutant no. 1, *Cla*I (lane 1), *Pst*I (lane 2), *Eco*RI-*Hind*III (lane 3); lanes 4-6: Chromosomal DNA from putative *C. glutamicum glnA* mutant no. 2, *Cla*I (lane 4), *Pst*I (lane 5), *Eco*RI-*Hind*III (lane 6); lanes 8-10: wt *C. glutamicum* chromosomal DNA, *Cla*I (lane 8), *Pst*I (lane 9), *EcoRI-Hind*III (lane 10). **B**: Autoradiograph of fragments of lanes A: 1-12 hybridized with a *glnA*-specific probe. Lanes correspond in both panels.

Integration of the recombinant plasmid into the *C. glutamicum* chromosome (and resultant disruption of the *glnA* gene) was selected for on LA containing 50μg/ml Km and 10% sucrose. The mating experiment yielded two putative *C. glutamicum glnA* disruption mutants (Km-resistant, sucrose-sensitive). Chromosomal DNA was extracted

from both putative mutants, digested with selected restriction enzymes and probed with a *C. glutamicum glnA*-specific probe by Southern hybridization (Fig. 4.6). The fragments detected by Southern hybridization are represented diagrammatically in Fig. 4.7, and confirm integration of pK19*glnA* into the *glnA* gene of *C. glutamicum*. The confirmed *C. glutamicum glnA* mutants were designated GLNA19-1 and GLNA19-2.

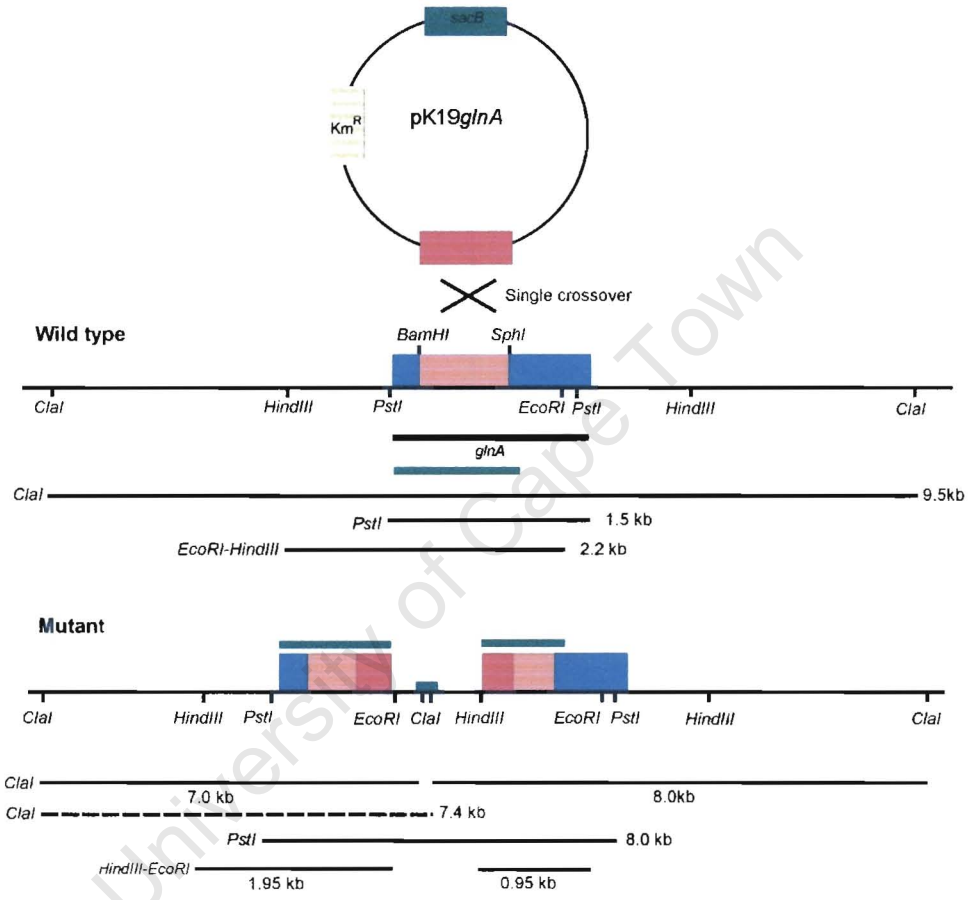



**Fig. 4.7**. Diagrammatic representation of pK19*glnA* integration into the *C. glutamicum* chromosome and the chromosomal DNA restriction enzyme fragments of wild type and mutant strains. Green bars indicate regions to which the *glnA*-specific probe hybridizes. The dotted line represents a fragment which is the result of partial enzyme restriction in mutant no.1 (see Fig. 4.6). The red region indicates the *glnA* region cloned into pK19*mobsacB*. The *glnA* gene in the *C. glutamicum* wild type chromosome is indicated by the black bar. The above diagram is not to scale.

### 4.3.3 Construction and confirmation of *hkm* disruption mutant

The *hkm* sequence used to construct the replacement vector for disruption of this gene was obtained from pGOGup. The construction of pGOGup is described in section 4.2.4. *C. glutamicum* mutants in *hkm* were generated in essentially the same way as the *glnA* mutants although the mating protocol was slightly different (see Methods). Selection of the mating mixture on medium containing 50μg/ml Km and 10% sucrose resulted in the identification of several putative *hkm* mutants. Restreaking on the selective medium reduced this number to one putative mutant (Km-resistant, sucrose-sensitive).

To confirm disruption of *hkm*, chromosomal DNA was extracted from the putative mutant, digested with selected restriction enzymes and probed with *a C. glutamicum hkm*-specific probe by Southern hybridization (Fig. 4.8). Restriction sites for *EcoRV* flank the *hkm* gene in the *C. glutamicum* wild type genome, and Southern hybridization with an *hkm*-specific probe detected a ~10kb *EcoRV* fragment (Fig. 4.8, B5).



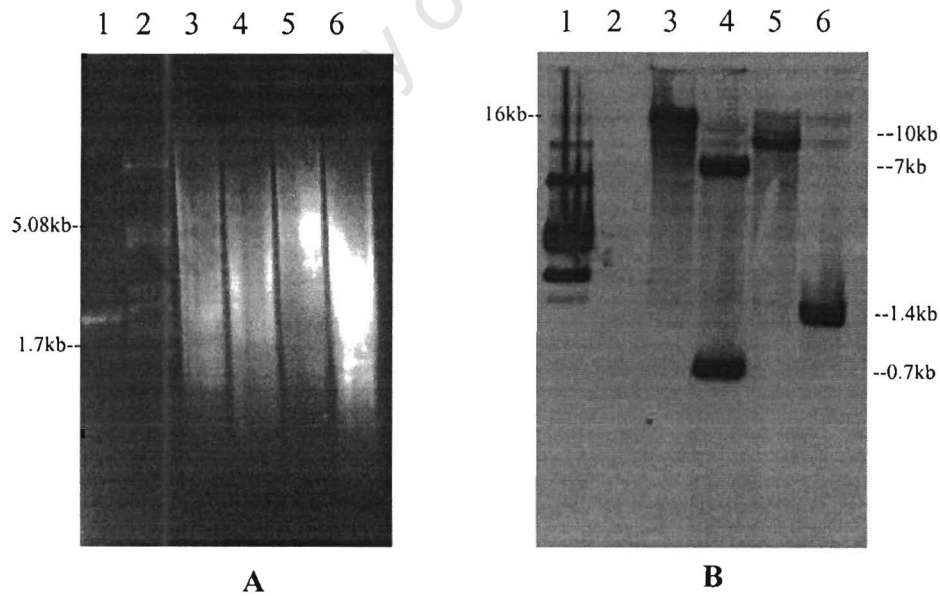



**Fig. 4.8** Southern hybridization to confirm inactivation of the *C. glutamicum hkm* gene. **A:** Agarose gel electrophoresis of DNA digested with selected restrictions enzymes. Lane 1: 50ng pGOGup; Lane 2: λ-*Pst*I molecular marker; Lane 3: Chromosomal DNA from putative *hkm* disruption mutant, *EcoR*V; Lane 4: Chromosomal DNA from putative *hkm* disruption mutant, *Sal*I; Lane 5: Chromosomal DNA from *C. glutamicum* wild type, *EcoR*V; Lane 6: Chromosomal DNA from *C. glutamicum* wild type, *Sal*I. **B:** Autoradiograph of lanes A: 1-6 hybridized with an *hkm*-specific probe. Fragment sizes shown are approximate.

The corresponding fragment in the putative mutant was ~16kb in size (Fig. 4.8, B3), confirming integration of the ~6kb replacement vector pK19*hkm* into this region of the chromosome. Similarly, *SalI* restriction sites also flank *hkm* such that a ~1.4kb *SalI* fragment was detected by Southern hybridization in the wild type (Fig. 4.8, B6). In the case of the putative mutant, a ~7kb as well as ~0.7kb fragment was detected (Fig. 4.8, B4). The additional fragment in the mutant is the result of a *SalI* restriction site that occurs within pK19*hkm*. The confirmed *C. glutamicum hkm* mutant was designated HKM19.

### 4.3.4 Growth studies of mutants

Preliminary investigations of *C. glutamicum glnA*$^-$ and *hkm*$^-$ strains involved studying their growth on various nitrogen sources. GLNA19-1 and HKM19 were inoculated onto MM plates containing 2mM $NH_4Cl$, 100mM $NH_4Cl$, urea (2% w/v), glutamine, glutamate, asparagine, aspartate, proline, histidine or arginine as the sole nitrogen source (all amino acids at 20mM). GLNA19-1 was unable to grow on any nitrogen source except glutamine, while HKM19 grew on all nitrogen sources.

To determine whether a second GS enzyme may be active in *C. glutamicum* under different culture conditions, GLNA19-1 was grown in 2mM and 100mM $NH_4Cl$ liquid MM with aeration (Fig 4.9). As expected, growth rates were much higher in nitrogen rich than nitrogen poor medium. However, GLNA19-1 was again unable to grow without added glutamine, irrespective of nitrogen status, aeration and culture conditions. Enzyme assays revealed that strain GLNA19-1 had no detectable levels of GS activity, while GOGAT activity was unaffected in this mutant (results not shown).

Although HKM19 was able to grow on all tested nitrogen sources, we wanted to determine whether *hkm* disruption affected growth rates in different nitrogen sources. HKM19 was therefore grown in liquid MM with selected nitrogen sources, and the growth profiles were compared to that of the wild type.

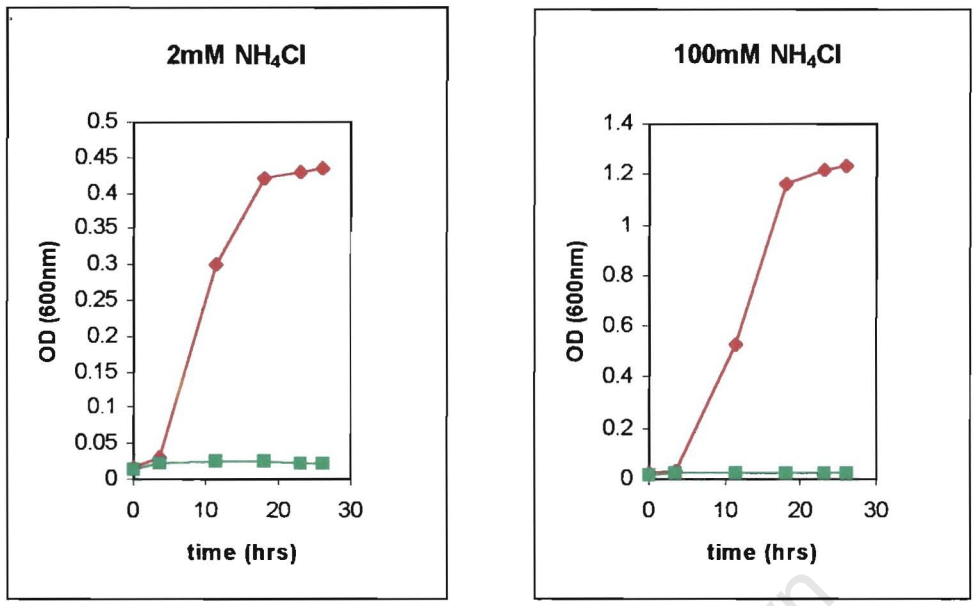



**Fig. 4.9** Growth of *C. glutamicum* GLNA19-1 in MM with 2mM or 100mM $NH_4Cl$ and with or without 20mg/ml glutamine (red and green, respectively).



The growth rates and profiles of HKM19 and the wild type were virtually identical in all nitrogen sources tested (Fig. 4.10). Both *C. glutamicum* strains grew well in $NH_4Cl$ and glutamine, while growth rates were poor in glutamate and in particular proline.

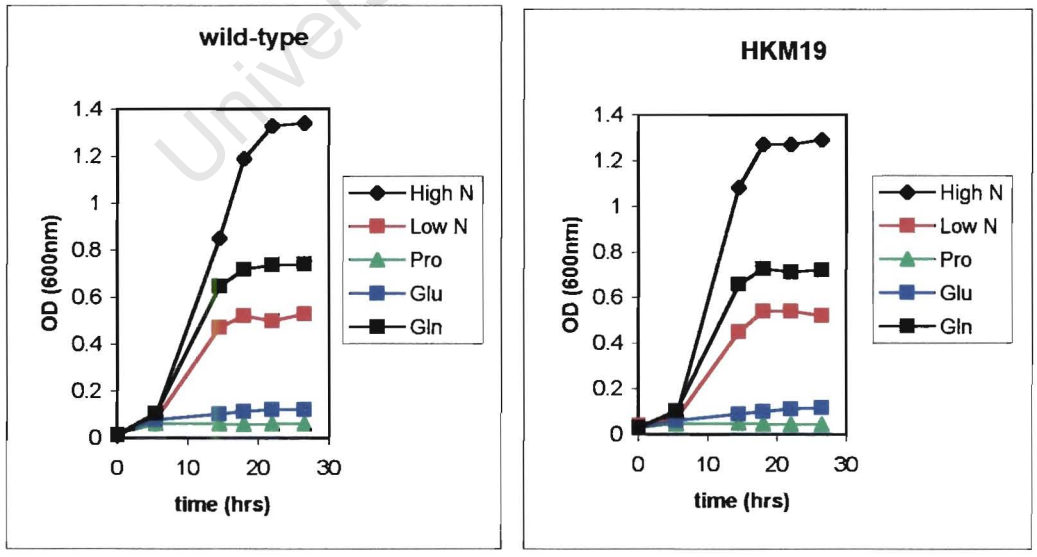



**Fig. 4.10** Growth of *C. glutamicum* wild type and HKM19 strains in MM with various nitrogen sources. High N: 100mM $NH_4Cl$; Low N: 2mM $NH_4Cl$. Amino acids were used at 20mM.

### 4.3.5 GS and GOGAT activities of HKM19

To determine the effect of *hkm* inactivation on GS and GOGAT activities in *C. glutamicum*, assays were performed on HKM19 and activity profiles were compared to that of the wild type strain. As before, enzyme activities were determined after growth in nitrogen-rich and nitrogen-limiting MM with either 2% or 0.05% glucose as the carbon source. GS assays showed that disruption of *hkm* does not affect the regulation or activity of GS in *C. glutamicum* to any significant extent; the pattern of activity was identical to that of the wild type *C. glutamicum* (Fig. 4.11).

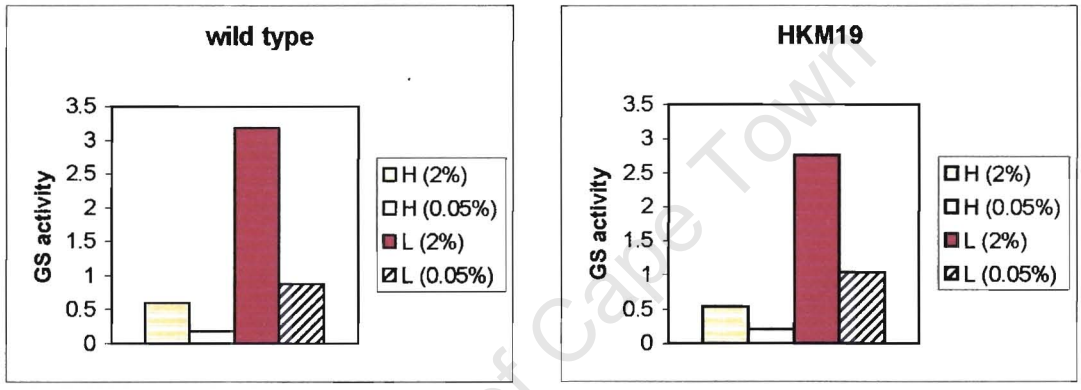



**Fig. 4.11** GS activities of *C. glutamicum* strains grown in various media. H: 100mM $NH_4Cl$; L: 2mM $NH_4Cl$ MM. Glucose concentrations are indicated in parentheses. Activities for the wild type are taken from the results in Chapter 3 (Fig. 3.1 & 3.2) and displayed for comparative purposes. GS activity is defined as μmol γ-glutamylhydroxamate $min^{-1}$ $mg^{-1}$ protein.

Since *hkm* is situated directly upstream of *gltBD*, we wanted to determine whether disruption of this gene would have an effect on GOGAT activity in *C. glutamicum*. Assays revealed that the *hkm* mutant does indeed have altered GOGAT activity profiles (Fig. 4.12). GOGAT activity under nitrogen-rich conditions with 2% glucose is approximately two-fold higher in HKM19 mutant than in the wild-type strain. When the glucose concentration is low, however, HKM19 displays very low GOGAT activity in nitrogen-rich conditions.

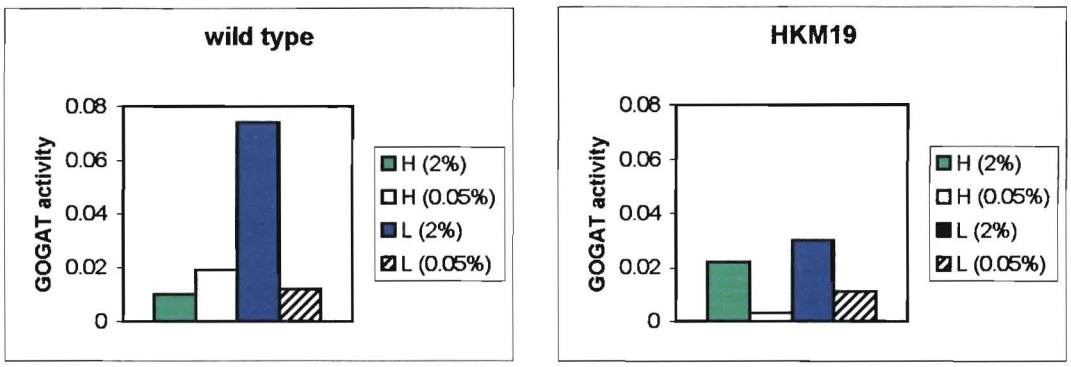



**Fig. 4.12** GOGAT activities of *C. glutamicum* wild type and HKM19 strains grown in various media. H: 100mM $NH_4Cl$ MM; L: 2mM $NH_4Cl$ MM. Glucose concentrations are indicated in parentheses. Activities for the wild type are taken from the results in Chapter 3 (Fig. 3.1 & 3.2) and displayed for comparative purposes. GOGAT activity is defined as μmol NADPH oxidized $min^{-1}$ $mg^{-1}$ protein.

In nitrogen-limiting medium with 2% glucose, the GOGAT activity of the mutant is less than half of that found in the wild-type strain under the same conditions. However, in nitrogen-limiting medium with 0.05% glucose, GOGAT activity of the wild type and HKM19 are essentially the same.

### 4.3.6 RNA hybridizations

Since the primary regulation of GOGAT activity is at the transcriptional level, we wanted to confirm the results of the above GOGAT assays with RNA studies. Total RNA was extracted from *C. glutamicum* wild type and HKM19 which were grown in nitrogen-rich and nitrogen-limiting MM (both with 2% glucose), and hybridized with a *gltBD*-specific probe. Densitometer readings of RNA hybridization signals indicated that *gltBD* transcription in HKM19 was reduced ca. 30% in nitrogen-limiting conditions in comparison to the wild type (Fig. 4.13). In nitrogen-rich conditions, however, *gltBD* transcription was essentially the same in both *C. glutamicum* strains.

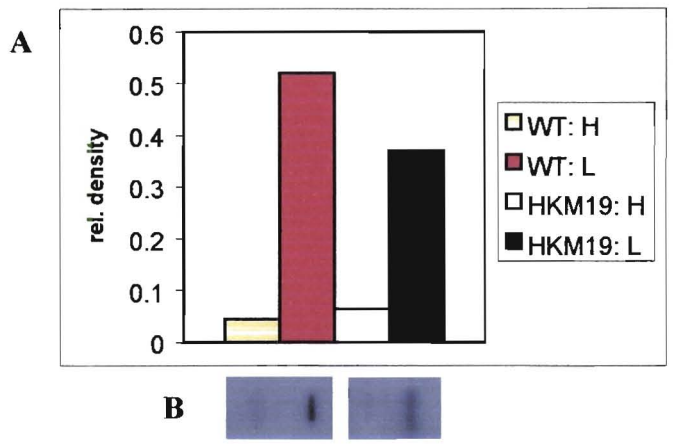



**Fig. 4.13 A.** Densitometric values of RNA hybridizations of *gltBD* transcripts of *C. glutamicum* wild type (WT) and HKM19. Strains were grown in MM with 2mM $NH_4Cl$ (L) or 100mM $NH_4Cl$ (H). Represented densities are relative to the background of the autoradiograph. **B**. Corresponding autoradiograph of hybridizations.

### 4.3.7 Activity of *hkm* promoter

To determine whether *hkm* was nitrogen-regulated, a DNA fragment containing the putative promoter of this gene was cloned into the CAT reporter plasmid pEKplCm. This recombinant plasmid was expressed in *C. glutamicum* grown in high and low nitrogen MM. CAT assays revealed that the *hkm* promoter has low activity and is only moderately elevated in low nitrogen conditions; CAT activities were 0.009 units $mg^{-1}$ in high nitrogen and 0.013 units $mg^{-1}$ in low nitrogen.

## 4.4 Discussion

Gene disruption techniques have been described previously in coryneform bacteria. Schwarzer and Pühler (1991) reported inactivation of the *C. glutamicum lysA* gene, while Reyes *et al.* (1991) disrupted the *gdhA* gene of *B. lactofermentum* and the *gltA* gene of *C. melassecola*. This genetic tool was later refined in *C. glutamicum* by Schäfer *et al.* (1994) who constructed specialized vectors for efficient gene disruption. Integration of the replacement vector by homologous recombination occurs either via a Campbell-like mechanism or a double crossover event (Vertès *et al.*, 1993). In our studies, we opted for mutants with single crossover inactivations since we used both Km-resistance and sucrose sensitivity as markers to ensure mutant integrity. When HKM19 and GLNA19-1 were grown in both complete and minimal medium with Km, there was some excision of the *sacB* gene such that the strains became sucrose-resistant but were still Km-resistant. However, this excision generally occurred at a frequency of less than $10^{-5}$. Schäfer and colleagues (1994) had similarly low excision frequencies in their work (~$10^{-4}$). Sucrose-sensitivity and Km-resistance of our mutants were always confirmed prior to and at the conclusion of each experiment.

Disruption mutants of *glnA* were generated in a different manner to the *hkm* mutants (see methods). While the general protocol was similar, the method used to generate HKM19 was refined. In addition to using several recipient cultures for *hkm* mutant generation (compared to just one in the *glnA* protocol), this method was also more precise in terms of cell numbers, culture volumes and general experimental procedure. The use of eight different recipient cultures seems to be an important step in the new mating protocol since the mating is very dependent on the state of the recipient culture. Slight variations in growth stage, aeration, and nutrient status seem to greatly affect conjugation frequencies. Using eight different cultures increases the chances of obtaining a culture in the required state. In the refined mating protocol, *C. glutamicum* recipient cells were heated to 48.5°C for 9 minutes. The temperature and exposure time is important since an increase in either leads to reduced cell viability and hence conjugation (Schäfer *et al.*, 1994). Elevated temperatures may increase fertility in two ways: 1) by directly inactivating the temperature-sensitive restriction enzymes that

degrade foreign DNA, or 2) indirectly as part of the SOS response (Schäfer *et al.*, 1990).

Growth studies of GLNA19-1 revealed that this strain had an absolute requirement for glutamine when grown in MM. This mutant was, however, able to grown in YT medium without added glutamine. This medium contains both tryptone and yeast extract (Sambrook *et al.*, 1989) which are good sources of peptides and proteins (Prescott *et al.*, 1990). Catabolism of these macromolecules yields amino acids including glutamine (Voet and Voet, 1990). The fact that GLNA19-1 was unable to grow on a variety of nitrogen sources in both liquid MM and solid MM, strongly argues against the notion that a second GS enzyme may be present that is only active under certain conditions. Indeed, although *glnA2* encoding a putative GSII enzyme was recently discovered in *C. glutamicum*, deletion or over-expression of this gene did not affect cellular GS activity (Nolden *et al.*, 2001). It seems that *glnA2* may be an inactive evolutionary remnant.

The region of *hkm* targeted for inactivation starts ca. 130 bp upstream from the start of *gltBD* transcription and ca. 60 bp upstream of the AmtR binding site. It therefore seems unlikely that integration of the replacement vector would affect AmtR binding and/or transcription of *gltBD*. However, this possibility cannot be completely ruled out at this stage. In contrast to *glnA*, mutation of *hkm* did not have any obvious effect on growth in *C. glutamicum*, and this strain was able to utilize all tested nitrogen sources. Clearly, this gene is not essential for cellular metabolism. If *hkm* indeed plays a role in regulating cellular GOGAT activity, disruption of this gene would presumably have little or no effect on nitrogen metabolism. The reason for this is that glutamate synthesis can be catalyzed by the action of other enzymes including GDH, glutaminase and transaminase (Schreier, 1992). This hypothesis is corroborated by the finding that *glt* mutants of *C. glutamicum* are not glutamate auxotrophs (Beckers *et al.*, 2001). Mutation of *hkm* also had no effect on GS activity in *C. glutamicum*.

In contrast to GLNA19-1, HKM19 was able to grow on solid MM with all tested nitrogen sources. We used inorganic nitrogen ($NH_4Cl$), urea and a variety of amino

acids including glutamine, glutamate as well as those that are catabolized to glutamate such as proline, histidine, and arginine (Voet & Voet, 1990). Interestingly, although HKM19 was able to grow on MM plates with proline and glutamate, the strain displayed poor growth in liquid medium with these amino acids as nitrogen sources. In *C. glutamicum*, proline is one of the compatible solutes used to counteract the effects of hyperosmotic stress (Csonka, 1989). As a result, proline uptake systems are induced in response to high external osmolality (Peter *et al.*, 1998). It seems that the MM used in our studies did not provide an environment with sufficient hyperosmotic stress, and consequently proline uptake was poor. Presumably, the limited proline uptake on solid MM is adequate to support the slow bacterial growth rates. The uptake of glutamate in *C. glutamicum* occurs via a binding protein-dependent mechanism (Krämer and Lambert, 1990) that is regulated by intracellular pH and $K^+$ concentration (Krämer *et al.*, 1990). As is the case with proline, the conditions within the MM were seemingly not conducive to efficient glutamate uptake. In contrast to proline and glutamate, glutamine supports high growth rates and is efficiently transported into the bacterial cell via a sodium-dependent system (Siewe *et al.*, 1995).

The fact that *hkm* inactivation seems to reduce the expression of *gltBD* transcription in response to nitrogen starvation suggests that Hkm may be directly or indirectly involved in this induction. GOGAT activities of HKM19 grown in low nitrogen were reduced around 50% in comparison to the wild type, while transcription of *gltBD* in the mutant was only ca. 30% lower than the wild type under the same conditions. However, as mentioned in the previous chapter, Northern analyses cannot always accurately quantify transcription. Although we did not probe for a constitutive transcript (such as 16S rRNA) to accurately determine RNA concentration, we used spectophotometry in combination with gel analysis to obtain this information. Furthermore, we found that even when identical RNA concentrations of the same sample were loaded in different wells of the Slot Blot® apparatus, one sometimes obtained variable hybridization signals. Other researchers have had similar findings (Andreas Burkovski, Univ. of Cologne, Germany, personal communication). This phenomenon may be the result of variable binding of the RNA to the membrane and/or uneven hybridization of the probe. In conclusion then, such "slot blot" Northern hybridization analyses are useful for

*estimating* transcriptional induction and repression. Taking this into account, the RNA hybridization studies in HKM19 therefore show the same trend as the GOGAT assays. Both our enzyme assays and RNA analyses suggest that Hkm is somehow involved in the enhanced induction of GOGAT activity in response to nitrogen limitation.

Given the location of the AmtR binding site in the intergenic region between *gltB* and *hkm* (see Fig. 4.3), it was tempting to speculate that this repressor protein may also regulate the expression of *hkm*. In fact, if the putative –10 and –35 consensus regions of the *hkm* promoter (based on the consensus motifs suggested by Pátek *et al.*, 1996) were indeed located in the positions proposed, binding of AmtR might well prevent the RNA polymerase from attaching to the *hkm* promoter. Although the promoter region of *hkm* has not been determined, the *hkm* promoter fusion vector contains ~200bp upstream of *hkm* and ~70bp of the *gltB* coding sequence. Since the two genes have opposite orientations, the *gltB* sequence should have no effect on the activity of the *hkm* promoter in the fusion construct. CAT assays revealed that the activity of the *hkm* promoter was almost two orders of magnitude lower than that of *glnA* (see section 2.3.4). Also, there was no significant induction of *hkm* in response to low nitrogen. In fact, the moderate increase in *hkm* promoter activity in low nitrogen may be the result of a general cellular response to nutrient deprivation. It seems likely that this gene is expressed at a low, constitutive level, consistent with its putative function as a sensor protein. Thus, the *cat* fusion studies do not support the suggestion that *hkm* might be subject to regulation by the AmtR repressor protein.

The occurrence of a PAS core domain in the primary sequence of Hkm suggests that it may function as a sensor protein in a signaling pathway in *C. glutamicum*. PAS motifs have been identified in hundreds of proteins, and almost all of them are either known or putative receptors, signal transducers, or transcriptional regulators (Zhulin *et al.*, 1997). In fact, in members of the kingdoms *Archae* and *Bacteria*, the PAS domains occur almost exclusively in sensors of two-component regulatory systems. Furthermore, most PAS domains identified in prokaryotes occur in histidine kinases (Ponting & Aravind, 1997).

Two-component regulatory systems are characterized by consisting of a histidine kinase sensor protein and a response regulator (Appleby *et al.*, 1996). The N-terminal region of the histidine kinase senses stimuli, while the C-terminal region contains a conserved histidine residue that is autophosphorylated in response to the stimulus. The phosphoryl moiety is then transferred from the histidine of the sensor protein to a conserved aspartate in the receiver domain of the response regulator. The phosphorylated regulator is activated as a result of the phosphorylation and is able to interact with DNA or another signaling protein.

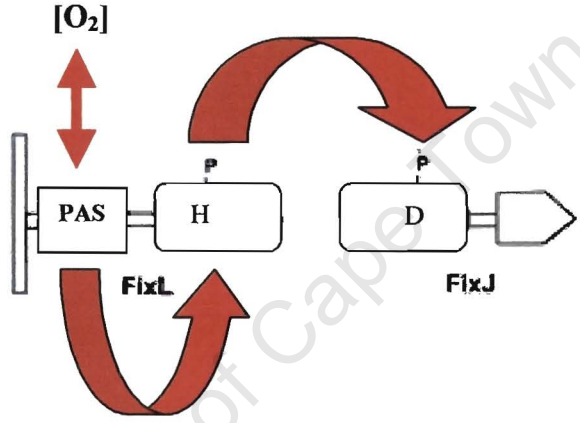



**Fig. 4.13** Phosphorelay in the FixL/FixJ system of *Sinorhizobium meliloti*. Phosphorylated histidine (H) and aspatate (D) residues are shown. See text for explanation. Adapted from Taylor and Zhulin (1999).



A well-studied example of a two-component regulatory system is the FixL/FixJ pathway of *Sinorhizobium meliloti* (David *et al.*, 1988). FixL is the sensor protein that is responsive to oxygen concentration. When oxygen concentrations are low in the cell, oxygen dissociates from the PAS motif in FixL (Gilles-Gonzalez *et al.*, 1994) causing a conformational change in this domain (Fig. 4.13). This change in turn alters the structure of the transmitter domain to increase autophosporylation of the conserved histidine residue. FixL then catalyzes the phosphoryl transfer from the histidine to the aspartate of the response regulator (Gilles-Gonzalez *et al.*, 1995). Phosphorylated FixJ functions as a transcriptional activator of the *nif* genes which are involved in nitrogen fixation.

Other PAS-containing sensors from two-component regulatory systems include the *E. coli* Aer aerotaxis protein which senses oxygen (Rebbapragada *et. al.* 1997), TodS of the *Pseudomonas* toluene-degrading pathway (Lau *et al.*, 1997), DctS of *Rhodobacter capsulatus* which senses energy levels (redox)(Hamblin *et al.*, 1993), NifL of *Azotobacter vinelandii* which responds to oxygen concentration and fixed nitrogen (Dixon, 1998), and KinA involved in sporulation initiation in *B. subtilis* (Ponting and Aravind, 1997). The role of PAS domains in the regulation of these and other two-component systems has been reviewed in detail by Taylor and Zhulin (1999).

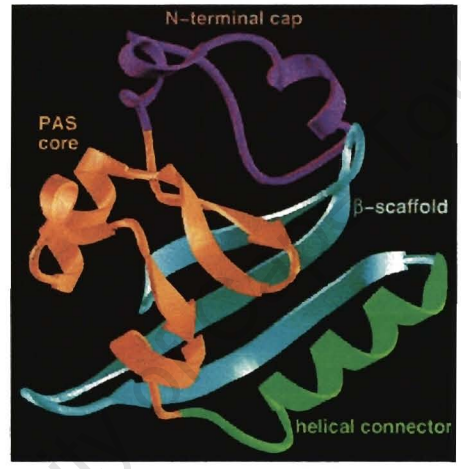



**Fig. 4.14** Proposed PAS three-dimensional structure of the *E. halophila* PYP sensor protein. The various regions consist of the following amino acids: N-terminal cap (purple), 1 to 28; PAS core (orange), 29 to 69; helical connector (green), 70 to 87; the β-scaffold (blue), 88 to 125. Taken from Taylor and Zhulin (1999).

Based on domain mapping and crystallographic analysis, Pellequer *et al.* (1998) proposed that the PYP protein contains a prototypical PAS domain. This hypothesis was supported by subsequent structure determination of the PAS domains in the FixL protein of *Bradyrhizobium japonicum* (Gong *et al.*, 1998) and the human HERG protein (Morais Cabral *et al.*, 1998). The PYP protein is derived from bacterium *Ectothiorhodospora halophila* and is a blue light receptor containing a number of α-helices and β-sheets (Fig. 4.14). The three regions characteristic of PAS domains are shown, as well as the variable N-terminal cap. The PAS core has the highest density of conserved residues, and contains the photosensing active site of PYP (Pellequer *et al.*, 1998).

Database searches have shown that there is considerable variation in PAS sequences of different organisms (Taylor and Zhulin, 1999). The Sll0799 protein from *Synechocystis* sp. has 5 PAS domains, where the three N-terminal ones most likely originated from a duplication event (Zhulin and Taylor, 1998). These multiple copies may serve to enhance the sensitivity of the protein to a specific stimulus. The other two PAS domains, located in the N-terminus, have different origins. Where different types of PAS domains are present in the same protein, each motif may be responsive to a different stimulus. Since Hkm in *C. glutamicum* contains only one PAS domain, this protein presumably responds to a single stimulus.

In summary, PAS domains are widespread in sensory proteins that respond to a variety of different stimuli. Hkm is a putative sensor protein that contains such a PAS motif, however lacks the other domains characteristic of a histidine kinase. It is therefore unlikely that Hkm could function as a histidine kinase in a two-component regulatory system. A very recent study by Little *et al.* (2002) on the *Azotobacter vinelandii* NifL-NifA regulatory system has shown that protein-protein interaction may be an important component of signal transduction. The NifL sensor contains a typical N-terminal PAS domain that is responsive to redox, while a protein-protein interaction with GlnK (a $P_{II}$ homolog) is mediated via the C-terminal region in response to nitrogen status. This interaction is dependent on $Mg^{2+}$, ATP and α-ketoglutarate. Like the *E. coli* $P_{II}$ protein, GlnK is uridylylated in response to nitrogen starvation. Under nitrogen sufficient conditions, unmodified GlnK directly interacts with NifL causing it to inhibit the transcriptional activation of NifA. During nitrogen starvation, the GlnK-UMP is unable to interact with NifL, and NifA is consequently free to activate transcription of the *nif* genes via a $\sigma^{54}$ RNA polymerase holoenzyme. Interestingly, the interactive C-terminal region of NifL resembles the transmitter domain of histidine kinases. However, no autokinase or phosphotransferase activity has been detected *in vitro* (Austin *et al.*, 1994).

In light of the above findings, an interesting possibility for Hkm function is that the PAS domain may indeed sense a stimulus (possibly nitrogen status), which would cause a conformational change in the protein. This would then allow Hkm to transmit this

signal to another protein via direct interaction. Other studies have shown that PAS domains add increased specificity and stability to protein dimers, in particular heterodimers (Pongratz *et al.*, 1998). The GOGAT enzyme in *C. glutamicum* is such a heterodimer, and nitrogen deprivation may activate Hkm to stabilize the GltB-GltD protein. An alternative suggestion is that Hkm may interact with AmtR and assist release of this repressor from the *gltBD* promoter during nitrogen limitation.

In conclusion then, various possibilities exist as to the putative role of Hkm in *C. glutamicum*. Our research suggests that Hkm appears to represent a fine-tuning regulatory component of GOGAT activity. Further work will be crucial in confirming the function of this protein in *C. glutamicum* metabolism.

The following and final chapter consolidates the research described in Chapters 2 to 4, and discusses them in the context of a regulatory model and proposed future work.

# Chapter 5

## Conclusions and Future Research

Nitrogen availability is one of the many nutritional constraints faced by microorganisms in their natural environment. Consequently, some bacteria have evolved global regulatory systems that allow them to adapt to these conditions and selectively use compounds that result in the highest growth rates. In *C. glutamicum*, nitrogen uptake and assimilation is under the control of such a regulatory network, and the AmtR repressor has been identified as the pivotal protein in this system (Jakoby *et al.*, 2000).

The first reports of a global nitrogen regulator in *C. glutamicum* were published approximately two years ago (Jakoby *et al.*, 2000), and several key studies have contributed to our current understanding of nitrogen assimilation in this organism (Jakoby *et al.*, 1999; Jakoby *et al.*, 2000; Nolden *et al.*, 2001; Beckers *et al.*, 2001). Based on this research, the following model for nitrogen regulation in *C. glutamicum* was proposed.



When *C. glutamicum* is grown in nitrogen-rich conditions, AmtR binds to a specific sequence (see section 1.7) in the promoter regions of *glnA*, *amt*, and the *amtB-glnK-glnD* and *gltBD* operons, and prevents transcription of these genes (Fig. 5.1). In addition, nitrogen sufficiency stimulates the UR activity of the UR/UTase enzyme such that unuridylylated $P_{II}$ is present. $P_{II}$ in turn stimulates the ATase to catalyse the adenylylation of GS, thereby inactivating the enzyme. The result is that the pathways for ammonium uptake, as well as glutamine and glutamate synthesis function at a low, basal level.

When nitrogen becomes limiting, however, AmtR dissociates from its binding sites resulting in the transcription of *glnA*, *amt*, and the *amtB-glnK-glnD* and *gltBD* operons. The UTase enzyme is stimulated to catalyse the addition of UMP groups to form $P_{II}$-UMP. This uridylylated protein activates the deadenylylation of GS, giving rise to the active form of the enzyme. High concentrations of Amt, AmtB, GOGAT and active GS

ensure that nitrogen assimilation functions at an enhanced level in order to rectify the intracellular nitrogen shortage.

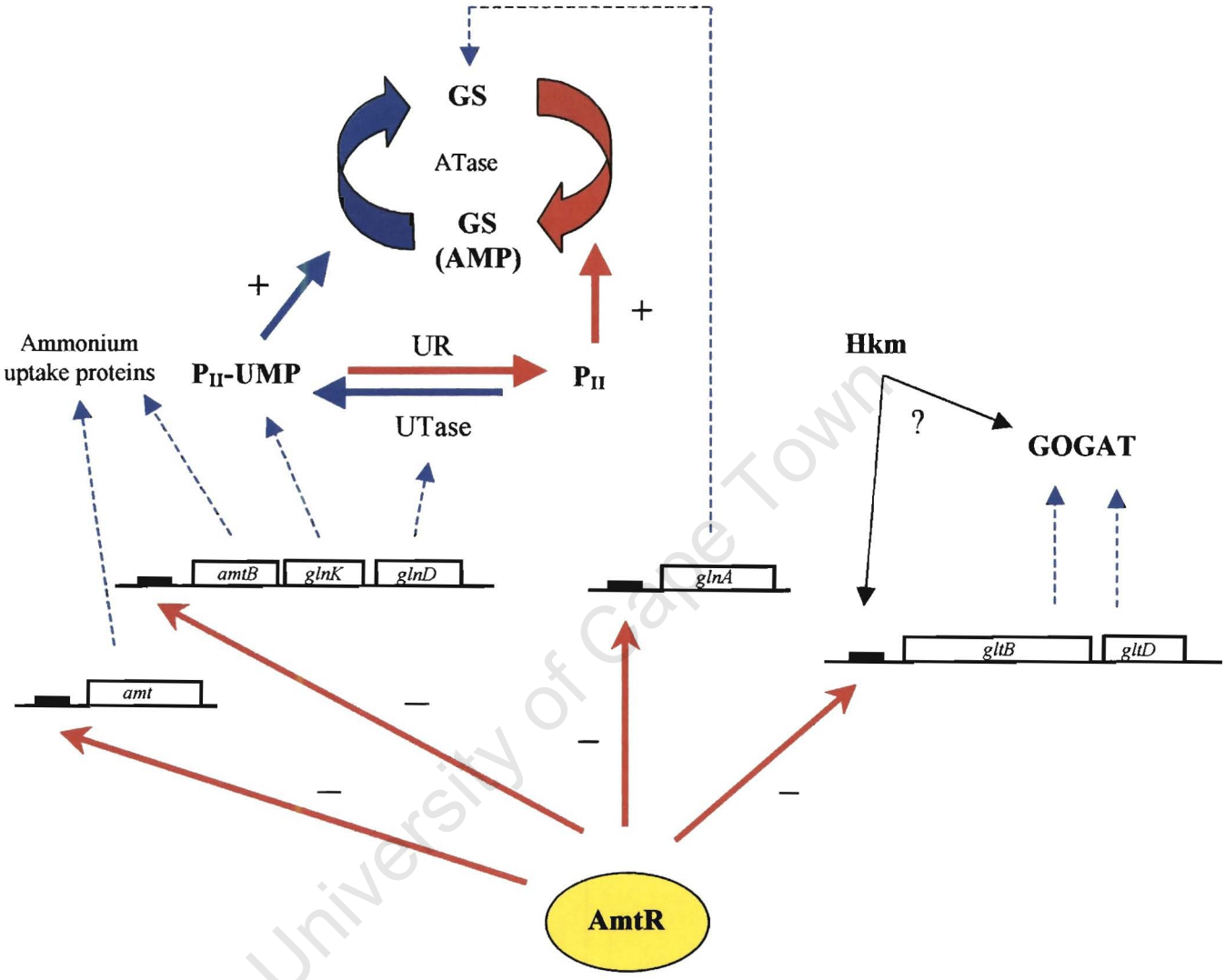



**Fig. 5.1** Regulation of nitrogen assimilation in *C. glutamicum*. Gene products are shown by dashed arrows. Nitrogen-rich conditions, red arrows; Nitrogen starvation, blue arrows. UR/UTase, uridylyl-removing enzyme/uridylyltransferase; ATase, adenylyltransferase. Activation and repression are indicated by + and –, respectively. ▬ represents AmtR binding sites. See text for explanation.

The results of the enzyme assays and RNA analyses performed in our studies are consistent with the above model. We have shown that transcription of both *glnA* and *gltBD* are greatly enhanced when AmtR repression is lifted during nitrogen starvation. As shown in Fig. 5.1, GS is the only nitrogen assimilatory enzyme that is able to respond rapidly to nitrogen depletion. Our SVPD studies as well as the characterisation

of *glnA* disruption mutants highlight the pivotal role of GS in cellular metabolism in *C. glutamicum*. Furthermore, our results strongly suggest that *glnA* is the sole gene in this organism whose encoded protein catalyses glutamine biosynthesis.

By virtue of the global AmtR repressor, the regulatory system in *C. glutamicum* clearly differs significantly from that in other Gram-positive bacteria such as *B. subtilis* (Fisher, 1999) and *S. coelicolor* (Wray and Fisher, 1993), and is perhaps more similar to that of *E. coli*. However, what makes nitrogen control in *C. glutamicum* unique is the fact that a single regulatory protein controls ammonium uptake, as well as the glutamate and glutamine biosynthetic pathways. In *E. coli*, for example, *glnA* transcription is regulated by the ntr response while *gltBD* transcription is subject to some other form of control (Grassl *et al.*, 1999). Similarly, in *B. subtilis*, *glnA* transcription is regulated by GlnR, while the *glt* genes are controlled by the GltC regulator. Furthermore, the global nature of AmtR is demonstrated by its ability to control GS at two levels. AmtR regulates transcription of 1) *glnA*, which affects cellular GS concentrations, and 2) of *glnK* and *glnD* which encode proteins involved in the posttranslational regulation of the GS enzyme.

As discussed previously, the *C. glutamicum* AmtR repressor protein exerts its control by binding to a specific sequence in the promoter region of the regulated gene (Jakoby *et al.*, 2000). In the case of *glnA*, the AmtR binding site is located between the –10 and –35 promoter regions, while for *gltBD* this site is located ca. 25bp upstream of the –35 region of *gltB* (see Fig. 4.3)(Beckers *et al.*, 2001). Although it is apparent how binding of AmtR to the *glnA* promoter excludes binding of the RNA polymerase, the mechanism of repression in *gltBD* is less clear. Evidently, sequences upstream of the –35 region are also involved in RNA polymerase binding.

Despite elucidation of the AmtR regulatory network, two critical questions remain unanswered. Firstly, how does AmtR respond to nitrogen status, and, secondly, what is the signal that causes release of this repressor from its binding site during nitrogen deprivation? Based on primary sequence analysis, it was concluded that AmtR belongs to the TetR (*E. coli* tetracycline repressor) family of transcriptional regulators (Jakoby

*et al.*, 2000). This protein has a helix-turn-helix motif that presumably constitutes the DNA binding region. Conserved domain searches (CD-Search, www.ncbi.nih.nlm.gov) showed that AmtR contains no other regulatory or enzymatic motifs, indicating that this repressor is not a response regulator of a typical two-component regulatory system. One possibility is that AmtR may interact directly with a sensory protein. Jakoby *et al.* (2000) suggested that $P_{II}$ may be a candidate for this type of interaction. In *E. coli*, the activity of $P_{II}$ is modulated by the synergistic binding of ATP and α-ketoglutarate (Atkinson and Ninfa, 1999). If the same holds true for *C. glutamicum*, then $P_{II}$ would essentially be the sensor protein that could relay energy and nitrogen status signals to AmtR.

Our preliminary studies of the PAS-containing Hkm protein suggest an involvement in the fine-tuning of GOGAT regulation in *C. glutamicum*. Considering its putative function as a sensory protein, Hkm could potentially transmit signals of nitrogen status to AmtR bound to the *gltBD* promoter. At this stage, it is important to note that AmtR *bound* to DNA is the molecule that is subject to a signal that causes its release from the promoter. Given the location and sequence variation in the AmtR binding site of *glnA* (Nolden *et al.*, 2001) and *gltBD* (Beckers *et al.*, 2001), it is conceivable that these bound repressors may have different conformations and may therefore be subject to modulation by different sensory proteins. Our studies suggest that Hkm affects *gltBD* but not *glnA* transcription, which support the above model. Therefore, $P_{II}$ may be required to lift AmtR repression of *glnA*, while Hkm may perform the same function to release AmtR from the *gltBD* promoter. An additional role for Hkm may involve stabilization of the GltB-GltD (GOGAT) heterodimer, since this type of interaction has been demonstrated in other proteins containing PAS domains (Pongratz *et al.*, 1998).

In light of the proposed sensory role for $P_{II}$, this protein may in fact be the crucial link between nitrogen assimilation and carbon metabolism. ATP concentration is a direct measure of the energy status of the cell (Tesch *et al.*, 1999), while α-ketoglutarate levels are an indicator of nitrogen availability (Woods and Reid, 1993). We have shown that high glucose concentrations increase *glnA* transcription in *C. glutamicum*, while *gltBD* transcription is unaffected. These findings indicate that AmtR repression of *glnA* is

lifted in response to glucose, while the same repressor remains bound to the *gltBD* promoter under the same condition. Perhaps Hkm is only able to sense nitrogen status and not carbon availability. Indeed, sensor proteins containing one PAS domain usually respond to only one type of stimulus (Taylor and Zhulin, 1999).

The results of our studies and the relevant cited work by other researchers clearly indicate that there is a link between nitrogen regulation and carbon metabolism in *C. glutamicum*. Physiologically, such a relationship would be advantageous as the bacterial cell would be able to coordinate central metabolism such that sufficient energy is provided for nitrogen assimilation.

Since the essential components of nitrogen assimilatory control in *C. glutamicum* have already been identified, future work needs to focus on the central protein involved in this system, namely AmtR. Considering the link between nitrogen and carbon metabolism, it would be interesting to establish whether AmtR may regulate the transcription of some genes involved in carbon metabolism. Construction and implementation of a two-hybrid system may prove useful to determine if $P_{II}$ is indeed able to interact with AmtR. In parallel with this, it will be important to establish whether the $P_{II}$ protein binds ATP and/or α-ketoglutarate, and whether its activity is subject to modulation by these molecules. Purification of the AmtR protein would allow crystallographic analysis which would in turn provide information as to the functional domains. Furthermore, co-precipitation experiments could be used to ascertain whether Hkm, or any other protein, is able to bind to AmtR. Similarly, purification of Hkm would allow refined studies for the determination of functional domains.

As far as the research described here is concerned, several avenues of investigation are still required in order to elucidate the role of Hkm in *C. glutamicum*. Probably the most important of these is generating an inframe deletion mutant of *hkm*. This would eliminate the possibility of any proximal effects that integration of the disruption vector may have on *gltBD* transcription. In addition to co-precipitation assays, purified Hkm could be used to determine whether this protein is capable of forming a protomeric molecule. This information may provide some insight into the function of the protein. In

the case of the human HERG protein, truncated subunits that have only the N-terminal PAS domain are able to form tetramers in solution (Li *et al.*, 1997). Other future work includes studying the possible interaction between Hkm and AmtR, and confirming the predicted absence of any enzymatic activity on the part of Hkm.

In conclusion, extensive research over the past few years has greatly expanded our knowledge of nitrogen assimilation and regulation in *C. glutamicum*. This work is of applied, as well as academic interest, since it may ultimately enable scientists to further manipulate amino acid excretion in industrially important strains.

# Appendix A

## Bacterial Strains and Plasmids Used

| Strain or plasmid | Relevant characteristics | Reference or source |
|---|---|---|
| **Strains** | | |
| *E. coli* JM1O5 | *supE endA sbcB15 hsdR4 rpsL thi* (*lac-proAB*), F' [ *traD36 proAB*$^{+}$ *lacI*$^{q}$ *lacZ* M15] | Yanisch-Peron *et al.* (1985) |
| *E. coli* JM109 | *recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* (*lac-proAB*), F' [ *traD36 proAB*$^{+}$ *lacI*$^{q}$ *lacZ* M15] | Yanisch-Peron *et al.* (1985) |
| *E. coli* DH5α | F' , *endA1 hsdR supE44 thi-1' recA1 gyrA96 relA1 deoR* (*lacZYA argF* ) U196 80d*lacZ* M15 | Grant *et al.* (1990) |
| *E. coli* DH5α*mcr* | *mcrA* ( *mrr hsdRMS mcrBC* ) | Grant *et al.* (1990) |
| *E. coli* S17-1 | Mobilizing donor strain; *hsdR pro recA* carrying RP4-2 Tc::Mu in the chromosome | Simon *et al.* (1983) |
| *C. glutamicum* ATCC 13032 | Type strain, $Nx^{R}$ | Abe *et al.* (1967) |
| *C. glutamicum* ATCC 14752 | Type strain; $Nx^{R}$ | A. Burkovski |

| | | |
|---|---|---|
| *C. glutamicum* RES167 | Restriction-deficient mutant of *C. glutamicum* AS019 | Liebl *et al.* (1991). |
| *C. glutamicum* GLNA19-1 GLNA19-2 | ATCC 13032 Δ*glnA*; $Nx^R$, $Km^R$ | This study |
| *C. glutamicum* HKM19 | ATCC 13032 Δ*hkm*; $Nx^R$, $Km^R$ | This study |

**Plasmids**

| | | |
|---|---|---|
| pBluescript SK | Cloning vector; $Ap^R$ | Stratagene Cloning Systems San Diego USA |
| pGS-2/71 | Bluescript (SK) derivative carrying the *C. glutamicum* ATCC 13032 *glnA* gene | C. Kenyon, AECI |
| pGSup21 | Bluescript (SK M13+) derivative carrying the 5' region and ~0.4 kb upstream of the *C. glutamicum glnA* gene | Schulz (1996) |
| pLEW146 | *Streptomyces* sp.-*E. coli* shuttle vector carrying the *Streptomyces coelicolor glnR* gene; $Ap^R$ | L. Wray |
| pDH18.1 | pUC derivative carrying the *Streptomyces viridochromogenes glnA* gene | W. Wohlleben |
| pDH19.2 | pUC derivative carrying the *Streptomyces viridochromogenes glnII* gene | W. Wohlleben |
| pTNC15 | Tn*5515* delivery plasmid; $Cm^R$ | A. Tauch |
| pCGL0040 | Tn*5531* delivery plasmid; $Km^R$ | J. Kalinowski |

| | | |
|---|---|---|
| pK18*mobsacB* | pK18 derivative carrying the RP4*mob* region and a modified *sacB* gene from *Bacillus subtilis*; $Km^R$ | Schäfer *et al.* (1994a) |
| pK19*mobsacB* | pK19 derivative carrying the RP4*mob* region and a modified *sacB* gene from *Bacillus subtilis*; $Km^R$ | Schäfer *et al.* (1994a) |
| pJC1 | *E. coli-C. glutamicum* shuttle vector; $Ap^R$, $Km^R$ | Cremer *et al.* (1990) |
| pEBM3 | Mobilizable *E. coli-C. glutamicum* shuttle vector; $Km^R$, CmR | Schäfer *et al.* (1994b) |
| pWK0 | *E. coli-C. glutamicum* shuttle vector; $Km^R$ | Schäfer *et al.* (1994b) |
| pEKplCm | Promoterless *cat*, *ori* of pBL1; $Km^R$ | A. Burkovski |
| pGOGup | pGEM™ cloning vector (Promega) carrying ~0.5kb of the *C. glutamicum gltB* gene and ~0.5kb upstream | This study |
| pK19*glnA* | pK19*mobsacB* containing internal fragment of *C. glutamicum glnA* | This study |
| pK19*hkm* | pK19*mobsacB* containing part of *C. glutamicum hkm* gene | This study |

$Ap^R$, ampicillin resistance; $Nx^R$, nalidixic acid resistance; $Km^R$, kanamycin resistance; $Cm^R$, chloramphenicol resistance

# Appendix B

## DNA Techniques

### Electroporation of intact *C. glutamicum* cells

*C. glutamicum* cells were electroporated using a modification of the method used by Liebl *et al.* (1989). A *C. glutamicum* O/N culture was prepared in LM (see 2.2.1) supplemented with 50µg/ml Nx. The O/N culture (10 ml) was inoculated into 400ml LM with 0.5% glycine and 50µg/ml Nx, followed by incubation at 30°C with shaking. After reaching an optical density ($OD_{578}$) of 0.4, the cells were chilled in a mixture of ice and water for 10 mins and subsequently harvested by centrifugation (10,000 rpm, 10 min, 4°C). The cells were then resuspended in 100ml ice-cold buffer (10% w/v glycerol, 8 mM Tris-HCl, pH 7.4) and reharvested. The washing steps were repeated twice more. Residual supernatant was removed, and the cells were resuspended in 0.4ml ice-cold 10 % w/v glycerol. Cells were then dispensed into pre-cooled Eppendorf tubes in 100µl aliquots and stored at 70°C. Electroporation: Frozen cells were thawed on ice, washed twice with 20 volumes of ice-cold 10 % glycerol and resuspended in 98µl 10 % w/v glycerol. Plasmid DNA (in 2µl $dH_2O$) and 98µl cells were mixed and then dispensed into a cold electroporation cuvette (Bio-Rad, 2mm electrode gap). The cuvette was tapped on a bench top to remove air bubbles and pulsed with a Bio-Rad 'Gene Pulser™' using the 'Pulse Controller'. Electrical conditions used were as follows: voltage 2.5kV, capacitance 25µF, parallel resistance 600Ω. Immediately 1 ml BHIS was added to the electroporation cuvette, and the cell suspension was subsequently transferred to an Eppendorf tube. Cells were then incubated at 30°C with shaking for 2h and subsequently plated onto the appropriate medium with antibiotic selection. Plates were incubated at 30°C.

### Extraction of *C. glutamicum* chromosomal DNA

*C. glutamicum* was inoculated into 100ml LM containing 0.5% glycine and incubated with vigorous aeration at 30°C O/N. Cells were harvested by centrifugation (10,000 rpm, 10

min) and the pellet was resuspended in 10ml suspension buffer (10mM Tris-HCl, 1mM EDTA, 0.35M sucrose, pH 8.0). Dry lysozyme was added to 10mg/ml and the suspension was incubated at 37°C for 2h. An equal volume (10ml) of 2x lysing solution (100mM Tris-HCl, 20mM EDTA, pH 8.0) and 5ml of 5M $NaClO_4$ was then added. The mixture was heated at 55°C for 2h or until it became translucent and viscous. Hot (55°C) DNA phenol (15ml) was added and mixed gently to form a uniform emulsion. The mixture was then centrifuged (12,000 rpm, 10 min, RT) and the aqueous layer was retained. If the phenol was found to form a layer above the aqueous one, approximately 10ml TE (10mM Tris-HCl, 1mM EDTA, pH 8.0) was added and the emulsion and centrifugation steps were repeated. The aqueous layer was phenol-extracted twice more and then decanted into a glass beaker. Isopropanol was then added (0.6 volumes) and swirled to mix. The nucleic acid precipitate was held back with a Pasteur pipette and the lysate-isopropanol solution was poured off. Ethanol (75%) was added (20ml) and after 10 min the nucleic acid pellet was retained again and dried. The nucleic acid was then dissolved (which often took several hours) in 20ml TE buffer containing 0.1mg/ml RNase A. A 1h incubation at 37°C followed. The solution was extracted with 5ml chloroform:isoamyl (24:1) and the aqueous layer retained. Sodium acetate (0.1 volumes of 3M) was added, mixed with the aqueous layer after which 2 volumes of 95% ethanol was added. A glass rod was used to mix the solution and collect the precipitating DNA. Swirling was continued until both phases were completely mixed. The rod was placed in 30ml 70% ethanol for 10min after which the DNA was briefly air-dried (on the rod). The DNA was then dissolved in 1-3ml TE, which often took several days to resuspend.



**Extraction of *C. glutamicum* plasmid DNA**

A 10ml O/N culture was prepared in LB (Sambrook *et al*, 1989) with 0.5% glycine. Cells were harvested by centrifugation (10,000 rpm, 10 min), and all medium was removed from the pellet. Cells were then resuspended in 200µl ice-cold alkaline lysis solution 1 (50mM glucose, 10mM EDTA, 25mM Tris-HCl, pH 8.0) containing 15mg/ml lysozyme. The suspension was transferred to a 2ml Eppendorf tube and incubation at 37°C for 1h followed. Freshly prepared alkaline lysis solution 2 (400µl; 0.2M NaOH, 1% SDS) was

added, and the tube was gently inverted several times to allow mixing. The tube was incubated on ice for 5 min. Ice-cold potassium acetate pH 4.8 (400µl) was added, followed my mixing and incubation on ice for another 5 min. The suspension was then centrifuged at 4°C for 30 min (14,000 rpm). After centrifugation, the supernatant was transferred to a fresh tube. An equal volume of phenol/chloroform was added, followed my vortexing for 10 seconds and centrifugation at room temperature for 6 min. The top phase was transferred to a fresh tube and re-extracted with chloroform:isoamyl alcohol (24:1). The supernatant (500µl) was removed to a new tube, and two volumes 100% alcohol were added. The mixture was incubated on ice for 10 min and subsequently centrifuged (4°C, 14,000rpm). The pellet was washed with 70% ethanol, dried and resuspended in 30ml TE containing 1mg/ml RNaseA.

**"10 minute" small-scale plasmid DNA extraction (*E. coli*)**

TENS buffer was made up fresh (in the following order):

| Stock | (in 10 ml) |
|---|---|
| $dH_2O$ | 9.58 ml |
| 25% SDS | 200 µl |
| 10 N NaOH | 100 µl |
| 1 M Tris-HCl (pH 8.0) | 100 µl |
| 0.5 M EDTA | 20 µl |

An O/N cell culture was prepared in 2 ml YT broth (Sambrook *et al.*, 1989) in a 2 ml Eppendorf tube. Cells were collected by microfugation for 30 s. The supernatant was then poured off, and the cell pellet was resuspended in 300 µl TENS buffer (cell suspensions not to be left in TENS longer than 10 min). Sodium acetate pH 5.4 (150 µl of 3M solution) was added, the lysate was briefly vortexed, and subsequently microfuged for 5 min. The supernatant was then removed to a new tube, and microfuged for another 5 min. The supernatant was again removed to a new tube, 0.9 ml 100% ethanol was added, and the tube was placed at -20°C for 10 min (but not more than 15 min). Microfugation followed at 4°C, and the DNA pellet was washed with 70% ethanol, airdried, and resuspended in 30µl sterile $dH_2O$.

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