GENETIC AND BIOCHEMICAL STUDIES
OF *Vibrio alginolyticus*
GLUTAMINE SYNTHETASE

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SUBMITTED IN FULFILMENT OF REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

IN THE FACULTY OF SCIENCE
UNIVERSITY OF CAPE TOWN
CAPE TOWN

OCTOBER 1988

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CERTIFICATION OF SUPERVISOR

In terms of paragraph eight of "General regulations for the degree of Ph.D." I, as supervisor of the candidate, R. Maharaj, certify that I approve of the incorporation in this thesis of material that has already been published or submitted for publication.

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ACKNOWLEDGEMENTS

I wish to express my appreciation to my supervisors, Prof Dave Woods for expert and enthusiastic guidance and Prof Frank Robb for valuable suggestions and encouragment during the course of this study.

I am also thankful to members of the Department of Microbiology for sharing their ideas and expertise. Special thanks to Dr Sue Robb and Wyn Jones for assistance with DNA sequencing and to Dr Ralph Kirby for help with DNA sequence analysis.

Many people have assisted with the preparation of this thesis and I would like to thank the following:

Willie van der Westhuisen for the use of his computer.

Zeenid Hay who willingly made available her typing skills.

Morag Kenmuir, Vishu Lakhoo, Nehru Pillay and Yachika Reddy for assistance with the preparation of figures.

Fiona Hughes, Greg Blatch, Michelle Babaya and Dr Nozi Mjoli for proof-reading.

Thanks are also due to family and friends for their kindness and moral support.

I am indebted to my sister Savi who taught me the meaning of the word altruism.

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ABSTRACT

A genomic library of the collagenolytic Vibrio alginolyticus strain was established in Escherichia coli HB101 employing the positive selection vector pEcoR251. A glutamine synthetase (GS) gene, glnA was identified by complementation of the glnA deletion in E. coli ET8051 glnA, glnL, glnG deletion strain. The glnA region of V. alginolyticus was cloned on a 5.7 kb insert in pRM210.

GS synthesis in V. alginolyticus was regulated by temperature, oxygen and nitrogen availability. The cloned V. alginolyticus glnA gene was expressed in E. coli from a promoter contained within the cloned fragment. V. alginolyticus glnA expression in E. coli from pRM210 was subject to regulation by temperature, oxygen and nitrogen levels. GS specific activity in an E. coli wild-type strain was not affected by temperature or oxygen. The expression of GS on pRM211, a deletion derivative of pRM210, was not regulated by temperature, oxygen or nitrogen levels in E. coli. Plasmids pRM210 and pRM211 enabled an E. coli glnA, glnL, glnG deletion mutant to utilize arginine or low levels of glutamine as nitrogen sources. In contrast, transactivation of histidase production in the E. coli glnA, glnL, glnG strain containing the Klebsiella aerogenes hut operon did not occur. E. coli glnF deletion strains containing pRM210 or pRM211 were unable to grow in minimal medium containing arginine as the sole nitrogen source. The cloned V. alginolyticus DNA fragment was able to activate

expression of the cloned E. coli glnA gene in trans, under conditions of limiting nitrogen. The E. coli glnL, glnG region cross hybridized with a fragment of DNA located downstream of the glnA structural gene in pRM210.

The nucleotide sequence of a 3.5 kb fragment containing the V. alginolyticus glnA and glnL genes was determined. The glnA and glnL genes occupied regions of 1.4 and 1.0 kb respectively, with a 0.75 kb intergenic region. The upstream region of the glnA gene contained tandem promoters. The upstream promoter resembled the consensus sequence for E. coli σ^{60} promoters whereas the presumptive downstream promoter showed homology with Ntr regulated promoters. Expression of glnA from the upstream promoter produced a transcript with an untranslated leader sequence of 267 nucleotides. Four putative NR_I binding sites were located between the tandem promoters. The glnL gene was preceded by a single putative NR_I binding site. A third ORF was located 45 bp downstream from the glnL gene. The amino acid sequence deduced from this sequence was homologous to the N terminal end of the E. coli NR_I protein. The deduced amino acid sequences of the V. alginolyticus glnA and glnL genes were analyzed and compared with the reported amino acid sequences of GS and NR_{II} proteins from other organisms. The deduced amino acid sequences of the V. alginolyticus glnA and glnL genes shared 89 and 53% homology with the E. coli glnA and glnL genes respectively. It is concluded that the V. alginolyticus GS structural gene forms part of a glnALG

operon with components that are functionally analogous to the *E. coli* *glnL* and *glnG* genes.

ABBREVIATIONS

A-bis-A	Acrylamide-bis-Acrylamide
Ap	ampicillin
bp	base pair(s)
BSA	bovine serum albumin
Cm	chloramphenicol
CTAB	hexadecyltrimethyl-ammonium bromide
dNTP	deoxyribonucleotide triphosphate
DPC	diethylpyrocarbonate
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetate
ELISA	enzyme-linked immunosorbent assay
EtBr	ethidium bromide
GDH	glutamate dehydrogenase
GOGAT	glutamate synthase
GS	glutamine synthetase
γ-GT	γ-glutamyl transferase
h	hour(s)
IPTG	isopropylthiogalactoside
LA	Luria agar
LB	Luria broth
min	minute(s)
M_r	molecular mass
Nif	nitrogen fixation
Ntr	nitrogen regulated
ORF	open reading frame
p	plasmid
PAGE	polyacrylamide gel electrophoresis

PEG	polyethylene glycol
RF	replicative form
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	second(s)
Tc	tetracycline
UT	uridylyltransferase
UV	ultraviolet
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER ONE

GENERAL INTRODUCTION

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GENERAL INTRODUCTION

1.1 *Vibrio alginolyticus*

The *V. alginolyticus* strain is an aerobic, halotolerant, collagenolytic, Gram-negative bacterium isolated from a batch of South African cured hides (Welton and Woods, 1973; 1975). The isolate was initially classified as *Achromobacter iophagus* and subsequently reclassified as the marine bacterium *V. alginolyticus* by M. Hendrie of the National Collection of Industrial Bacteria, Aberdeen, Scotland. The ability of *V. alginolyticus* to utilize sucrose has been employed to distinguish it from the closely related *V. parahaemolyticus*, the causative agent of food-borne gastroenteritis (Shewan and Ve'ron, 1974).

This strain is of interest because it produces an inducible extracellular collagenase with the highest reported specific activity (Lecroisey *et al*., 1975), during the stationary phase of growth (Reid *et al*., 1978; 1980). In addition to the collagenase, six alkaline serine exoprotease enzymes (Hare *et al*., 1981; 1983; Deane *et al*., 1986) and an SDS-resistant, Ca2+-dependent, alkaline serine exoprotease (Deane *et al*., 1987) are produced during the stationary growth phase. Production of collagenase and exoprotease is repressed by nitrogen levels, for example high ammonia or the addition of one of several amino acids to the medium represses exoprotease production (Reid *et al*., 1978; Hare *et*

al., 1981). An interesting aspect of the production of the collagenase and the serine proteases is that their synthesis is specifically regulated by temperature and oxygen (Hare et al., 1981). Collagenase and protease production are inhibited by a temperature shift from 30 to 37^{0}C and by a lack of oxygen whereas macromolecular synthesis is not affected.

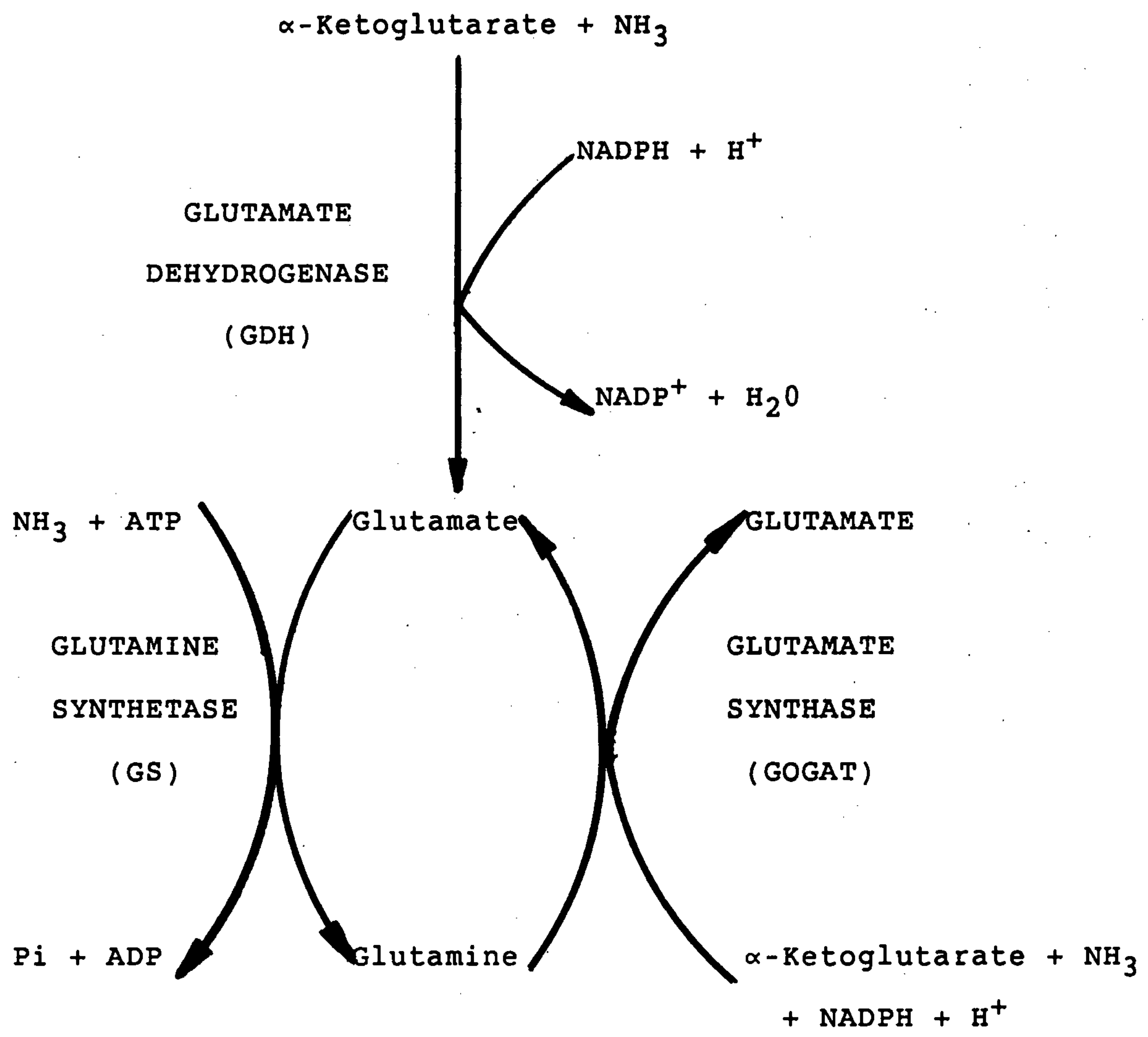
Members of the family Enterobacteriaceae do not generally export soluble proteins; secreted proteins either remain in the periplasmic space, or are integrated into the inner or outer cell membrane (Michaelis and Beckwith, 1982; Silhavy et al., 1983; Oliver, 1985). Recent evidence however, indicates that the enteric bacterium Serratia marcescens does produce true extracellular proteins (Yanagida et al., 1986; Ball et al., 1987). Other reports of extracellular protein secretion in Gram-negative bacteria include members of Vibrionaceae (Howard and Buckley, 1985) and Pseudomonaceae (Lory et al., 1983).

1.2 Nitrogen assimilation in bacteria

1.2.1 Metabolic importance of glutamate and glutamine.

Apart from atmospheric nitrogen (N_2), ammonia is the most widespread nitrogenous compound on earth. It is the preferred nitrogen source for many prokaryotes, fungi and plants and the end product of nitrogen metabolism in many organisms. The two main routes for the assimilation of ammonia in bacteria are the direct reductive amination of α-ketoglutarate, catalyzed by glutamate dehydrogenase (GDH)

(EC 1.4.1.4), and an energy dependent coupled system involving glutamine synthetase (GS) (EC 6.3.1.2) and glutamate synthase (GOGAT) (EC 1.4.1.13) (Tempest et al., 1970)



Glutamate is an important intermediate in ammonia assimilation. It provides part of the carbon skeleton for the amino acids of the "glutamic acid family" and furnishes the amino group of others by transamination. Glutamate is also the precursor of glutamine, whose amide group provides some of the nitrogen atoms of amino acids and of purine and pyrimidine nucleotides (Fig 1.1). Thus, the combined action

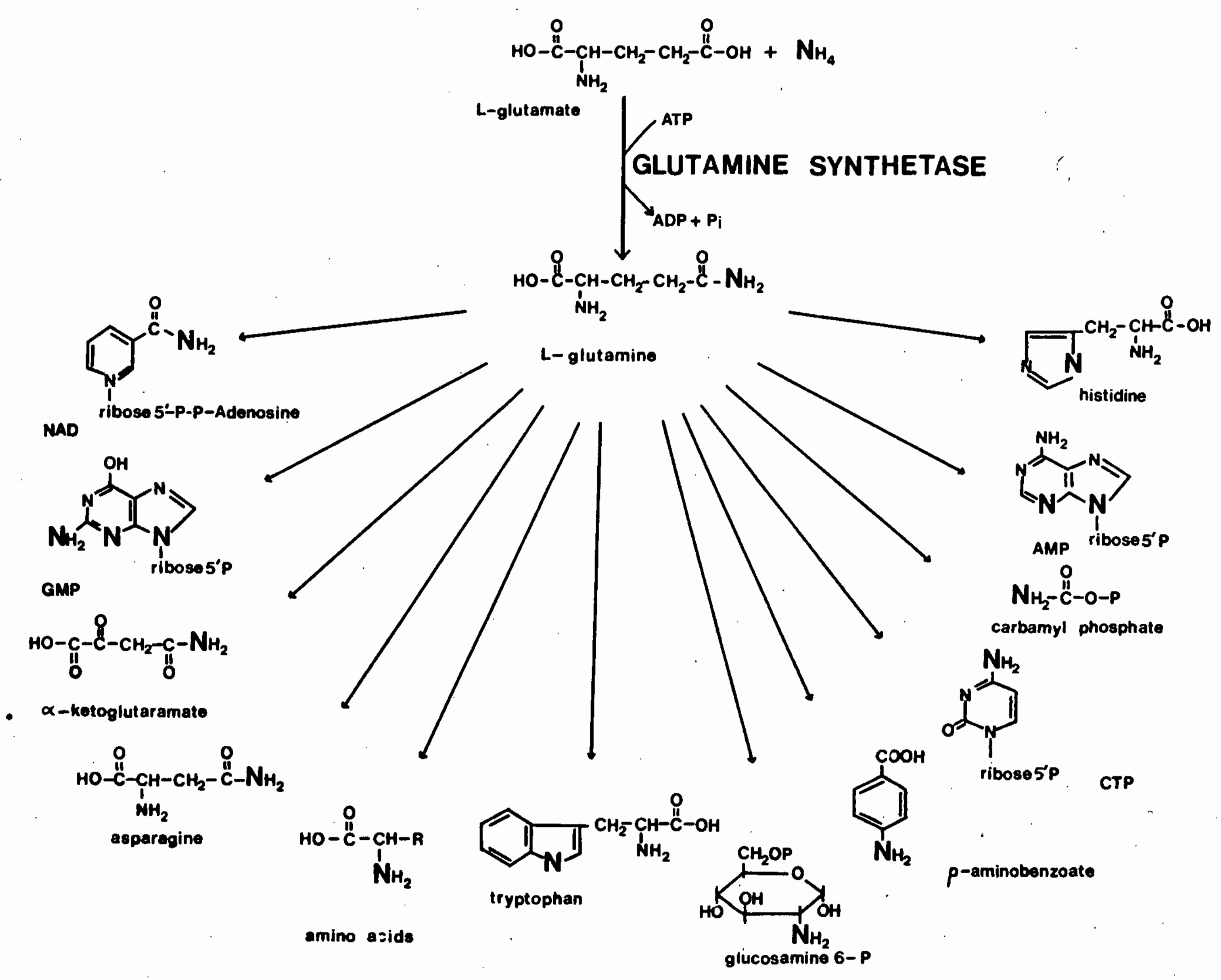


Fig. 1.1. Metabolic fate of glutamine

of GDH and GS serves to link carbohydrate and energy metabolism with nitrogen metabolism and is a branch point between synthetic and degradative pathways.

GS plays a major role in nitrogen assimilation when the extracellular concentration of ammonia is below 1 mM. Under these conditions glutamate is formed by the combined action of the GS and GOGAT enzymes. The GS/GOGAT pathway is ATP dependent and physiologically irreversible (Tempest et al., 1970). GDH catalyzed glutamate production has an unfavourable equilibrium for glutamate formation at low ammonia concentrations and is favoured during conditions resulting in low ATP levels (Tempest et al., 1970).

GS has been the subject of investigation in numerous biological systems as it plays a pivotal role in nitrogen metabolism. Regulation of the enzyme has been extensively studied in the Gram-negative bacteria Escherichia coli, Klebsiella pneumoniae and Salmonella typhimurium.

1.2.2 The GS enzyme. GS in E. coli and several other Gram-negative bacteria (Gancedo et al., 1968; Tronick, 1973) is composed of twelve identical subunits arranged in two hexagonal rings. The twelve subunits are arranged as the carbon atoms in two face-to-face benzene rings (Stadtman and Ginsburg, 1974). The GS subunit has a M_r of 52 kD and is one of the most complex and largest proteins to be examined by X-ray crystallography to date (Almassy et al., 1986). The divalent cation Mg^{2+} (or Mn^{2+}) is required for enzyme

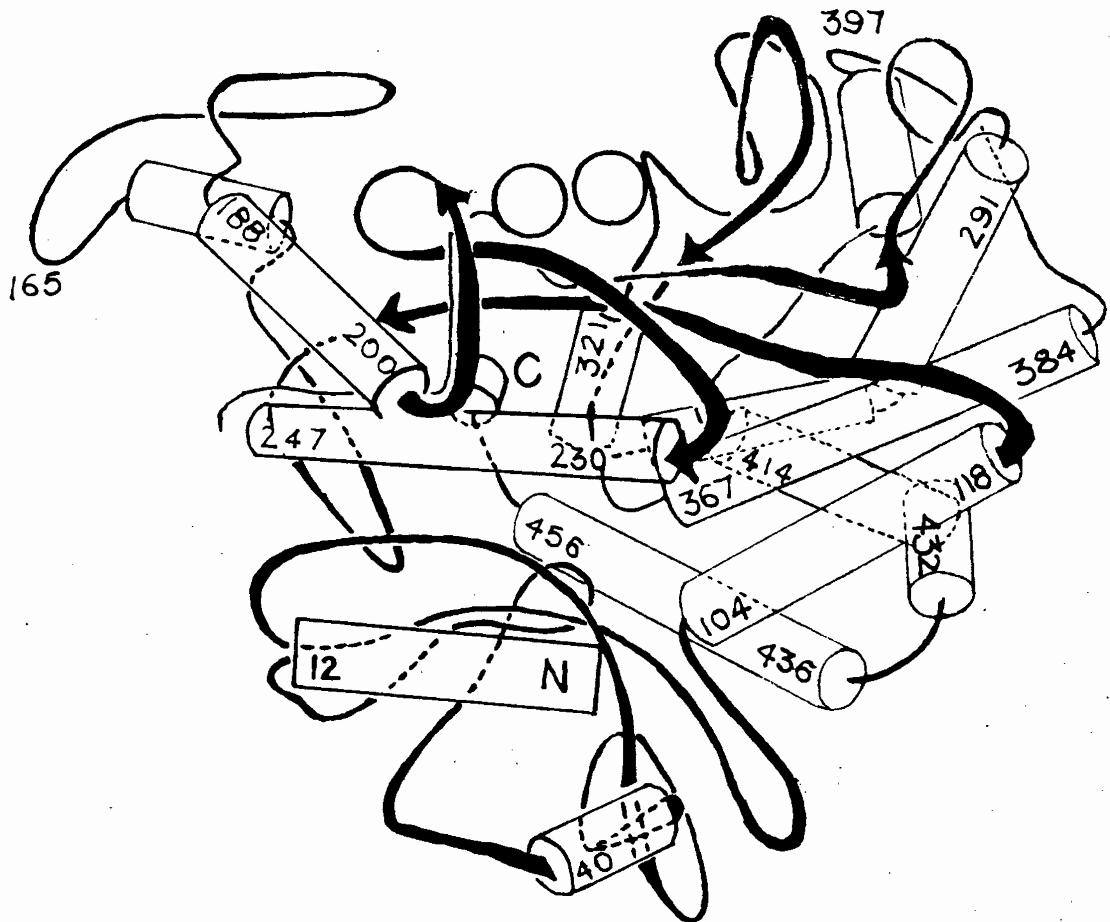


Fig. 1.2. A schematic drawing of the GS polypeptide chain (Almassy et al., 1986). The 6-fold axis is indicated by a hexagon at the left, and the metal ions by two circles in the upper centre. Cylinders, α-helices; heavy arrows, six prominent β-strands surrounding the metal ions. Bottom left, the N terminal domain (residues 1-103). The junction between the N- and C terminal domain is the start of the third helix (residue 104). The central loop is at the upper left.

stability and occurs between subunits in the intact enzyme structure.

Folding of the polypeptide in a single subunit results in 25 turns and loops (Fig 1.2). The small N-terminal domain comprises six strands of antiparallel β-sheets and one large and one small α-helix. The C domain contains numerous α-helices (seven long and six short) and an extended β-sheet (six long and three short strands). The GS polypeptide differs from other enzyme domains with mixed α- and β-segments in that the β-strands are antiparallel and the helices run perpendicular to the β-strands. Six prominent β-strands from the C domain form a partial barrel around the two metal ions. The metal ions are connected to the protein backbone by four bonds. The view of GS down the 6-fold axis reveals a petal-shaped molecule with a central channel. A central loop formed by the hydrophilic segment of residues 156-173 protrudes into the central channel. Six active site channels are located roughly midway between the outer periphery of the GS molecule and the central channel. The two metal ions required for catalytic activity are on one side on the channel nearer to the C domain. The enzyme is unusual in that each active site is at the interface between two subunits. The GS active site resembles the antigen-binding sites of antibodies in that the surfaces are formed by two polypeptide chains, both having antiparallel β-structures. One surface of the GS active site is formed mainly by six β-strands of the C domain of one subunit and the other surface by two β-strands of the N domain of the

adjacent subunit.

1.2.3 Regulation of GS. GS in the enteric bacteria is subject to rigorous cellular control. The complex pattern of regulation and structure of the enzyme stem from its central role in nitrogen metabolism. GS activity and synthesis is regulated in response to the availability of the nitrogen source (for reviews, see Shapiro and Stadtman, 1970; Tyler, 1978; Magasanik 1982; Rhee et al., 1985; Magasanik and Neidhardt, 1987; Reitzer and Magasanik, 1987). The mechanisms of regulation of the enzyme include:

- the cyclic covalent modification of proteins
- interconversion of active (taut) and inactive (relaxed) forms of the enzyme
- cumulative feedback inhibition of enzyme activity
- irreversible inactivation and degradation of the enzyme.

Cyclic covalent modification. Transcriptional and post-translational control of GS in E. coli is mediated via an elegant cascade system involving interconversions of three proteins, as shown in Fig. 1.3 (Son and Rhee, 1987). The three cycles of covalent modification of proteins involve:

- the adenylylation and deadenylylation of GS (Fig 1.3. upper right cycle)
- the uridylylation and deuridylylation of Shapiro's regulatory protein, P_{II} (Fig 1.3, cycle on the left)
- the phosphorylation and dephosphorylation of NR_I (Fig. 1.3, lower right)

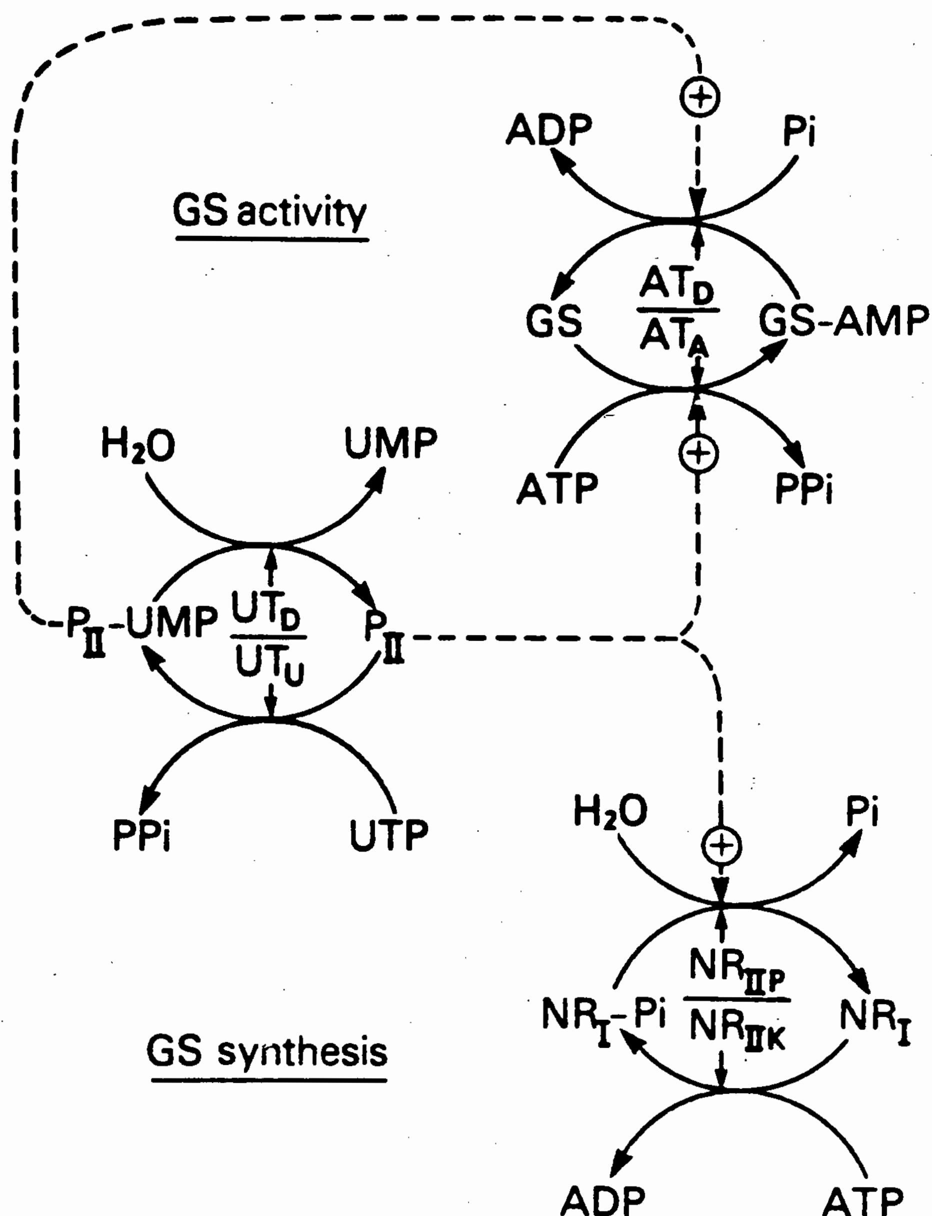


Fig. 1.3. Three protein interconversions involved in the regulation of GS in E. coli. AT_D/AT_A, adenylyltransferase (the glnE product) catalyzing the adenylylation (AT_A) and deadenylylation (AT_D) of GS; UT_D/UT_U uridylyltransferase (the glnD product) catalyzing the uridylylation (UT_U) and deuridylylation (UT_D) of P_{II} (the glnB product); NR_{IIP}/NR_{IIK}, the glnL product catalyzing the phosphorylation (NR_{IIK}) and dephosphorylation (NR_{IIP}) of the NR_I protein.

The key effectors in the cascade system are the intracellular concentrations and ratios of α-ketoglutarate and glutamine (Senior, 1975). In the presence of excess ammonia or glutamate, in glucose-limited cultures, GS is repressed and adenylylated (inactive). The adenylylation reaction involves the attachment of an adenylyl group from ATP, through phosphodiester linkage, to the hydroxyl group of Tyr^{397} in each subunit of the enzyme (Almassy et al., 1986). Because GS is composed of twelve identical subunits, up to twelve adenylyl groups can be attached to each enzyme molecule. Since adenylylated subunits are catalytically inactive under most physiological conditions, the specific activity of the enzyme is inversely proportional to the average number of adenylylated subunits per dodecamer. Deadenylylation is achieved by phosphorylysis of the adenyl-O-tyrosyl bond to yield ADP and unmodified GS (Anderson and Stadtman, 1970). The adenylylation (inactivation) and deadenylylation (activation) of GS are catalyzed at separate and essentially noninteractive sites (designated AT_A and AT_D respectively) on a single adenylyltransferase (Anderson et al., 1970; Hennig and Ginsburg, 1971; Rhee et al., 1978). The direction of catalysis of the adenylyltransferase is dictated by the regulatory protein P_{II} (Shapiro, 1969) thereby preventing a futile cycle of adenylylation and deadenylylation.

The activity of the P_{II} protein is modulated by interconversion between uridylylated and unmodified forms. In its unmodified form (P_{II}) this protein stimulates

adenylylation, and in its uridylylated form (P_{II}-UMP) it stimulates deadenylylation. Uridylylation of P_{II} involves the attachment of a uridylyl group from UTP, through phosphodiester linkage to the hydroxyl group of a specific tyrosyl residue in each subunit of the tetramer (Brown _et al_., 1971; Adler _et al_., 1975; Garcia and Rhee, 1978). A specific uridylyltransferase (UT_U) catalyzes transfer of the uridylyl group from UTP to each subunit of P_{II}. The deuridylylation reaction is catalyzed by a uridylyl-removing enzyme (UT_D) and involves the release of UMP from from P_{II}. Like the adenylylation/deadadenylylation reaction, both UT_U and UT_D activities reside in a single bifunctional polypeptide. The activities of UT_U and UT_D are controlled by the intracellular concentrations of glutamine and α-ketoglutarate which reflect the nitrogen status of the cell.

The P_{II} protein also plays an important role in the biosynthesis of GS. The gene encoding P_{II} has recently been sequenced and, although the complex functions carried out by P_{II} involve it in five separate protein/protein interactions, it is only 11,5 kD in size (Son and Rhee, 1987).

Activation of transcription of GS requires a phosphorylated NR_I protein (Hirschman _et al_., 1985; Reitzer and Magasanik, 1986). The phosphorylation-dephosphorylation of NR_I is catalyzed by the bifunctional enzyme NR_{II}. Dephosphorylation (inactivation) of $NR_I{\sim}Pi$ is activated by P_{II} (Ninfa and Magasanik, 1986). Thus the protein transmits

the metabolic information sensed by UT_U/UT_D to both a post-translational activity modulation system and a transcriptional regulatory system for GS.

Relaxed and taut forms of GS. The E. coli GS has an absolute requirement for the divalent cation Mg^{2+} or Mn^{2+} for enzyme stability in vitro. In the presence of Mn^{2+}, the enzyme is fully active and in a taut configuration. Taut GS is resistant to subunit separation by mild alkali or low concentrations of urea, has buried sulfhydryl and aromatic residues and assumes a compact configuration in solution. Extraction of the metal ions produces a relaxed enzyme that is catalytically inactive and susceptible to disaggregation. In the relaxed form, the protein has a less compact structure in solution and the sulfhydryl groups become accessible to protein denaturants. Reactivation of the relaxed GS in vitro is achieved by preincubation with either Mn^{2+}, Mg^{2+} or Ca^{2+} and is accompanied by burial of the exposed sulfhydryl groups and aromatic amino acids resulting in a tightened form of the enzyme. The tightened form of GS differs from the taut form in that it has a tendency to undergo intermolecular aggregation and crystallize out of dilute salt solutions (reveiwed by Shapiro and Stadtman, 1970).

Studies on the binding of divalent cations to GS have indicated that the unadenylylated (active) enzyme has a 10-fold higher affinity for Mn^{2+} in comparison with the adenylylated enzyme (inactive). The fact that divalent

cations play an important role in the structure and activity of GS suggests that the interconversion of a relaxed (inactive) and taut (active) form of GS in response to variations in concentrations of divalent cations may play a regulatory role in glutamine biosynthesis (Shapiro and Stadtman, 1970).

Cumulative feedback inhibition of enzyme activity. GS activity may be regulated through feedback inhibition by the multiple end products of glutamine metabolism (Stadtman and Ginsburg, 1974). They are CTP, AMP, glucosamine-6-P, histidine, tryptophan, carbomyl-P, alanine, glycine, and serine. Woolfolk and Stadtman (1964; 1967) have described the inhibition patterns observed with mixtures of the metabolites. The effects of the inhibitors were cumulative, provided that each inhibitor was present at a physiological concentration (Shapiro and Stadtman, 1970). This led to the suggestion that different metabolites bind to separate allosteric sites on the enzyme. Dahlquist and Purich (1975) proposed an alternative mechanism of inhibition in which all feedback inhibitors of GS bind in a competitive fashion with substrates at substrate binding sites. However, studies on the binding of feedback inhibitors to the unadenylylated GS indicate that the enzyme has separate sites for most of the feedback inhibitors (Ginsburg and Stadtman, 1973). Kinetic studies (Rhee *et al.*, 1985) suggest that GS contains a minimum of three binding sites for nonaromatic amino acids. In addition to the substrate L-Glu site, there are two allosteric sites for the binding of L- and D-amino acids.

The sensitivity of GS toward feedback inhibition varies with the adenylylation state of the enzyme (Ginsburg and Stadtman, 1973). The adenylylated enzyme is more sensitive to inhibition by AMP, L-tryptophan, L-histidine or CTP.

Oxidative inactivation of GS. GS is one of the enzymes turned over when cells are starved for nitrogen, suggesting its intracellular level is also regulated by proteolysis. As the enzyme has been shown to be susceptible to inactivation by a number of mixed-function oxidation systems (Levine *et al*., 1981; Oliver, 1981; Fucci *et al*., 1983; Levine, 1983a; 1983b; Roseman and Levine, 1987), it is believed that the oxidative inactivation of GS may constitute the "marking" step for intracellular GS turnover. The intracellular degradation of GS occurs in two steps. In the first step GS is "marked" by oxidative inactivation, followed by proteolytic digestion of the marked enzyme by a specific protease. Roseman and Levine (1987) have reported the isolation of a protease from *E. coli* which cleaves oxidised but not native GS.

The rate of oxidative inactivation of GS is both a function of the state of adenylylation and the degree of substrate saturation (Levine *et al*., 1981; Oliver, 1981). Binding of the substrate L-glutamate and some feedback inhibitors to the central loop (residues 156-173) of GS offer limited protection from proteolysis (Lei *et al*., 1979; Almassy *et al*., 1986). In the presence of ATP and glutamate, the physiologically active unadenylylated GS is protected from

inactivation, whereas the physiologically inactive adenylylated GS becomes more susceptible to oxidative inactivation. The physiological significance of this process is questionable, since the time required for the initial oxidation is a significant fraction of one generation (Levine et al., 1981).

Covalent modification of GS is perhaps the major modulator of enzyme activity. Adenylylation results in reduced binding affinity for divalent cations, increased sensitivity to feedback inhibitors, and greater susceptibility to proteolytic degradation, the net effect being reduced glutamine biosynthesis.

1.2.4 Genes involved in nitrogen assimilation. In the enteric bacteria, regulation of GS activity and expression involves the interaction of products of a number of genes. These are: glnB, glnD, glnE, glnF (ntrA), glnG (ntrC) and glnL (ntrB) (Reitzer and Magasanik, 1987). The nitrogen regulatory (Ntr) genes and their products are listed in Table 1.1. The structural gene for GS, glnA is part of the complex glnALG operon located at 86 min on the E. coli chromosome. It is transcribed counter-clockwise from glnA to glnG (Guterman et al., 1982; MacNeil et al., 1982; Pahel et al., 1982; Ueno-Nishio et al., 1983). Genes glnL and glnG constitute a Ntr locus designated glnR. Products of the glnF, glnL and glnG genes constitute the Ntr system. Genetic studies have revealed that the glnB, glnD, glnE and

Table 1.1. Glossary of gln genes and proteins. Adapted from Reitzer and Magasanik (1987)

Gene	Chromosomal location (min)	Peptide M_r (kD)	Product[a]	Target of glutamine synthetase regulation[b]
glnA	87	52	GS	—
glnB	55	44	P_{II}	CA, T
glnD	4	95	UTase/UR	CA, T
glnE		115	ATase	CA
glnF	70		$\sigma60$	T
glnG	87	110	NR_I	T
glnL	87	68	NR_{II}	T

Abbreviations:

[a] GS, glutamine synthetase

UTase/UR, uridylyltransferase/uridylyl-removing enzyme

ATase, adenylyltransferase.

[b] CA, catalytic activity; T, transcription

glnF genes occur at a number of distinct loci around the chromosome (Tyler, 1978; Magasanik, 1982).

glnA. Activation of expression of glnA, the structural gene for GS, requires the products of both glnG and glnF (Garcia et al, 1977; Kustu et al., 1979; Pahel and Tyler, 1979; MacNeil et al., 1982). Mutations in this gene result in glutamine auxotrophy (Gln^-) (Pahel and Tyler, 1979; MacNeil et al., 1982). Some of the mutations in this gene resulted in a thermolabile or enzymatically inactive enzyme (reviewed by Tyler, 1978; Magasanik, 1982). These mutants had the NtrC phenotype and the amount of GS antigen produced by the mutants under conditions of high and low nitrogen was comparable to that produced by wild-type E. coli grown in an ammonia-deficient medium.

glnB. The glnB gene is the structural gene of the regulatory protein P_{II}. The complete nucleotide sequence of this gene was recently determined by Son and Rhee (1987). The authors report the absence of an obvious binding site for RNA polymerase and suggest that the glnB gene is part of an as yet unidentified operon. The P_{II} protein consists of 103 amino acids. Two tyrosine residues reside at positions 46 and 51 respectively, where Tyr^{51} is the site of uridylylation. In K. aerogenes, glnB mutants with two different phenotypes have been isolated (Bueno et al., 1985). The glnB3 mutation (Foor et al., 1980) results in a phenotype similar to that of the glnD mutant of E. coli (Gln^-, Ntr^-, overadenylylated GS). These mutants produce an

altered P_{II} protein which retains the ability to stimulate adenylylation of GS but cannot itself be converted to a form capable of stimulating deadenylylation of GS. Insertions of transposons into the glnB gene produced strains which lacked P_{II} and produced high levels of GS even in the presence of excess ammonia. The adenylylation/deadenylylation of GS continued in the absence of P_{II}. However, the rate of the reactions catalyzed by the intracellular ATPase was reduced in comparison with cells possessing a functional glnB gene. These observations led to the proposal that P_{II} and not P_{II}-UMP was responsible for the decrease in GS levels in the presence of excess ammonia (Foor et al., 1980; Reuveny et al., 1981; Magasanik, 1982). Subsequent studies by Bueno et al. 1985) provide further evidence in support of this hypothesis. Under conditions of nitrogen excess, P_{II} apparently converts the product of glnL to a form that prevents the activation of transcription of the glnA gene.

glnD. The product of the glnD gene is the bifunctional enzyme uridylyltransferase. The native protein is a single polypeptide chain that undergoes slow irreversible aggregation during most steps of purification with a concommitant loss of activity (Garcia and Rhee, 1983). Insertions and deletions of the glnD gene confer a Gln^-, Ntr^- phenotype. In E. coli, mutants unable to produce UTase have an impaired ability to increase the level of GS in response to nitrogen deprivation. These mutants produce reduced levels of GS which is highly adenylylated (Bloom et al., 1978). Mutants of K. aerogenes lacking the glnD

product have a similar phenotype, except that their ability to increase the level of GS is not as severely affected (Foor *et al.*, 1978). The increase in the level of GS in response to nitrogen limitation does not occur in the *glnB*, *glnD* double mutant (Bueno *et al.*, 1985). Depriving the double mutant of the product of the *glnL* gene, restores its ability to produce high levels of GS during nitrogen limitation.

glnE. Mutations at *glnE*, the structural gene for adenyltransferase, result in the GlnC phenotype (Foor *et al.*, 1975). These mutants produce an ATase that has lost the ability to adenylylate GS. High levels of GS are produced by these cells under all growth conditions but the GS is only slightly adenylylated.

glnF. Activation of transcription of the *glnA* gene requires the product of *glnF* (Garcia *et al.*, 1977; Gaillardin and Magasanik, 1978; Hunt and Magasanik, 1985; Merrick and Stewart, 1985). The *glnF* gene is linked to the *argE* gene in *S. typhimurium* and with the corresponding *argG* gene in other enterics (Garcia *et al.*, 1977; Tyler, 1978). Strains with mutations in *glnF* are glutamine auxotrophs (Gln^-). These mutants are unable to derepress the synthesis of GS and have normal activities of all proteins involved in covalent modification of GS. In addition, expression of *glnF* is independent of the nitrogen status of the cell and is not controlled by the Ntr system (Garcia *et al.*, 1977; Gaillardin and Magasanik, 1978; Bruijn and Ausubel, 1983;

Merrick and Stewart, 1985). The product of the glnF gene has been recently purified and identified to be a σ factor specifically required for the transcription of Ntr and or nitrogen-fixation (Nif) promoters (Hunt and Magasanik, 1985). Studies with multicopy plasmids carrying glnF and rpoD suggest that the glnF product competes with the rpoD product (σ^{70} of RNA polymerase) in mediating transcription initiation by RNA polymerase at glnF-promoters (Merrick and Stewart, 1985). No significant DNA homology between glnF and rpoD has been detected employing Southern blotting. Further evidence that the product of the glnF gene is a σ factor stems from the in vitro transcriptional studies of Hirschman and co-workers (1985). Transcription of glnA by the glnF fraction was dependent on the addition of highly purified E. Furthermore, a partially purified glnF fraction which was not detectably contaminated with σ^{70} or $E\sigma^{70}$ allowed transcription of glnA from the major Ntr promoter. Hunt and Magasanik (1986) have therefore proposed rpoN as the alternate designation for glnF and σ^{60} its product.

glnG (ntrC). The glnG product (NR_I) is a bifunctional DNA-binding protein necessary for both positive and negative control of glnA expression (MacNeil et al., 1982; Pahel et al., 1982; Reitzer and Magasanik, 1983; Hirschman et al., 1985). The protein is dimeric and binds to specific sites in vitro (Reitzer and Magasanik, 1983; Ames and Nikaido, 1985; Hawkes et al., 1985). Synthesis of the glnG product is regulated in response to the availability of nitrogen in the growth medium, high-level expression of glnG occurs

under nitrogen-limiting growth conditions (Pahel *et al*., 1982). The activator function of the *glnG* product requires the *glnF* product and is apparently modulated by the *glnL* product (de Bruijn and Ausubel, 1983; Merrick and Stewart, 1985). The positive role of the *glnG* product in the regulation of *glnA* rests on the fact that any mutation which eliminates the *glnG* product fails to derepress *glnA*. GS production in these strains is unregulated and occurs at a low level. The suggestion of a negative role for the *glnG* product in *glnA* regulation is based on the presence in *glnG* both of mutations which confer *glnF* suppression and mutations which confer Gln^- phenotypes (Pahel and Tyler, 1979; MacNeil *et al*., 1982)

glnL (ntrB). The *glnL* product (NR_{II}) has been recently shown to act as an NR_I-kinase that can be converted by the *glnB* product (P_{II}) to an NR_I-phosphate phosphatase (Ninfa and Magasanik, 1986). Although the *glnL* product is not essential for the regulation of GS, it can strongly affect this regulation. Mutants that lack NR_{II} lack the ability to respond rapidly to ammonia starvation (McFarlane and Merrick, 1987). Strains which have a functional *glnL* gene but carry deletions or insertions in the *glnB* and *glnD* genes, are able to both activate and repress expression of *glnA*. This activation however, requires the products of *glnG* and *glnF* under all growth conditions. In mutants lacking the *glnB* product (P_{II}), the product of *glnL* can bring about the activation of synthesis of GS. Conversely, in strains lacking the *glnD* product (UTase), NR_{II} prevents

the activation of glnA expression (Bueno et al., 1985; Ninfa and Magasanik, 1986).

1.2.5 The glnALG operon. The complex glnALG operon of enteric bacteria can be transcribed from three promoters (Fig 1.4). Two tandem promoters are located upstream of the glnA structural gene (glnAp1 and glnAp2) (Reitzer and Magasanik, 1985; Leon et al., 1985) and a third one precedes glnL (glnLp) (Ueno-Nishio et al., 1983; Ueno-Nishio et al., 1984; Rocha et al., 1985). The E. coli glnALG operon occupies a region of approximately 4320 base pairs (bp), from which 1414 bp, 1050 bp and 1407 bp correspond to glnA, glnL and glnG coding regions respectively (Miranda-Rios et al., 1986).

glnAp2. The glnAp2 promoter (Fig. 1.5) lies closest to the glnA gene and constitutes the major Ntr promoter. Activation of transcription from this promoter has a requirement for core RNA polymerase complexed to σ^{60}, NR_1 and growth in nitrogen-deficient medium (Hirchman et al., 1985; Hunt and Magasanik, 1985). The nucleotide sequence of glnAp2 shares considerable homology with Ntr and Nif promoters (Ausubel, 1984). These promoters have a unique structure which is very different to the canonical sequence for prokaryotic promoters (Ow et al., 1983; Dixon, 1984). Such promoters lack the typical -10 and -35 regions found in most E. coli promoters. Instead, conserved regions of homology with the invariant dinucleotide GC at -12 and the invariant purine dinucleotide GG at -24 with respect to the

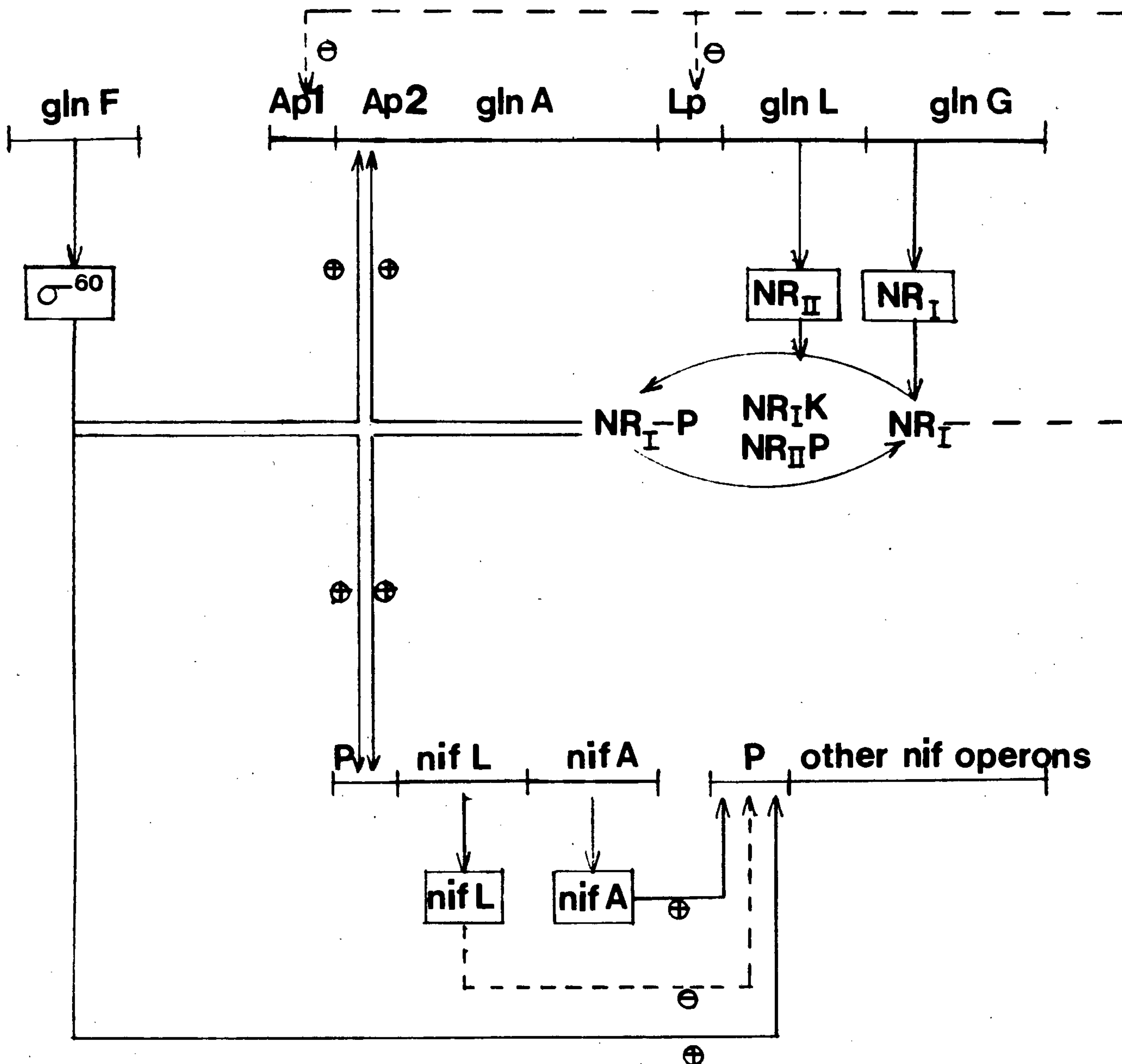


Fig. 1.4. Model for Ntr regulation of the glnALG and Nif operons. Adapted from Alvarez-Morales *et al*. (1984). +, activation; -, repressionts

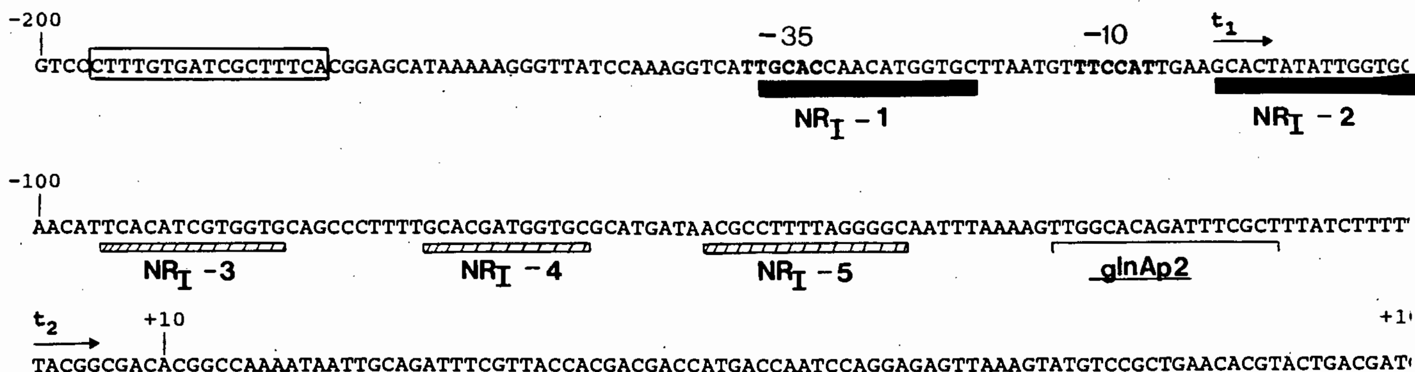


Fig. 1.5. Nucleotide sequence of the _E. coli_ _glnA_ promoter region (Reitzer and Magasanik, 1986). The start sites of transcription from _glnAp1_ and _glnAp2_ are designated as t_1 and t_2 respectively. The strong NR_I binding sites, 1 and 2, are identified by solid bars; the weak binding sites (3,4,5) are identified by dashed bars. The -10 and -35 regions of the _glnAp1_ promoter are in boldface type. The possible CAP recognition sequence is boxed. The coordinate +1 is defined as the first nucleotide of the t_2 transcript.

transcriptional start site are present. Promoters that are activated by the *glnF* product and either NR_I or the *nifA* product have the following consensus sequence **CTGGYAYRN4TTGCAN6-11+1** (Ausubel, 1984). In *E. coli*, the nucleotide sequence **TTGGCACAN4TCGCT** which occurs between base pairs -27 and -11 relative to the transcriptional start at +1 has been designated *glnAp2* (Reitzer and Magasanik, 1985). Deletion of a single bp of this sequence at position -27 eliminated transcription of *glnA* from this promoter (Reitzer and Magasanik, 1986). The σ^{60}-RNA polymerase complex has been shown to recognize and bind to this nucleotide sequence *in vitro* (Hunt and Magasanik, 1985).

glnAp1. The secondary promoter (*glnAp1*) for *glnA* transcription is located approximately 100 bp upstream of the Ntr promoter (Fig. 1.5). In contrast to *glnAp2*, this promoter has the canonical -10 and -35 regions (Reitzer and Magasanik, 1985) that characterize the Pribnow-type promoters recognized by RNA polymerases bound to the σ^{70} factor (Hawley and McClure, 1983; Reznikoff *et al*., 1985). Activation of transcription from the upstream promoter has an absolute requirement for the catabolite activator protein bound to cyclic AMP (cAMP) and does not require either the *glnF* or *glnG* product. The *glnAp1* promoter is subject to repression by NR_1, which activates transcription from *glnAp2* (Pahel and Tyler, 1979; Reitzer and Magasanik, 1985). This promoter is relatively weak and serves to maintain a low intracellular concentration of GS under conditions of carbon deficiency and nitrogen excess. The reduced strength of

this promoter results from the weak -10 and -35 RNA polymerase binding sites and a spacing of one more than optimal between the two sites (Reitzer and Magasanik, 1985):

	-35		-10
glnAp1	**TTGCAC**	**- N18 -**	**TTCCAT**
σ^{70} consensus	**TTGACA**	**- N17 -**	**TATAAT**

Both the -10 and -35 regions share 50% homology with the σ^{70} promoter consensus sequence. Initiation of transcription at glnAp1 is enhanced by increasing the homology of the -10 RNA polymerase contact site to the consensus sequence. Osorio et al. (1984) reported the isolation of a spontaneous, up-promoter mutation in E. coli (gln-76). This mutant produced high levels of GS in the absence of a functional glnG product under both nitrogen-limiting and nitrogen-excess growth conditions. A molecular characterisation of the gln-76 mutation by Leon et al (1985) revealed a single T-A transversion in the -10 region of glnAp1. Increased promoter strength was due to the introduction of one of the most conserved bases in the -10 region (Hawley and McClure, 1983):

glnAp1	**TTCCAT**
gln-76	**TACCAT**

Evidence in support of the higher transcriptional efficiency from the gln-76 promoter is provided by the primer extension experiments performed by Leon et al. (1985). The transcriptional start sites were identical in both the gln-76 and wild-type E. coli strains.

glnLp. The glnLp promoter is located approximately 250 bp downstream of the glnA gene (Fig. 1.6) and serves to maintain low intracellular levels of NR_I and NR_{II} in cells grown under conditions of excess nitrogen (Ueno-Nishio et al., 1984; Reitzer and Magasanik, 1985). In nitrogen replete cells, the ratio of GS to NR_I polypeptides is 80:1. When the carbon source becomes limiting, the ratio of GS to NR_I is reduced by a factor of 2 to 6. Therefore the initiation of transcription at this promoter is essential to maintain the basal levels of NR_I and NR_{II}.

This promoter has structural and functional similarities with the glnAp1 promoter. Both glnAp1 and glnLp are minor promoters of the glnALG operon, are repressed by NR_1 and have a recognizable canonical E. coli promoter sequence (Reitzer and Magasanik, 1983; Ueno-Nishio et al., 1984; Reitzer and Magasanik, 1985). While the -10 RNA polymerase recognition site at the glnLp promoter has excellent homology (100%) with the consensus sequence, the -35 region shares a relatively low degree of homology (50%). The -10 and -35 regions preceding the transcriptional start (Ueno-Nishio et al., 1984) are as follows:

-35 -10

TTCGCG - N19 - TATAAT

A single base transversion C.G to G.C in the -35 region resulted in an up-promoter mutation (Ueno-Nishio et al., 1984). In this mutant (pgln94) a HindIII linker has been positioned at a HhaI endonuclease cleavage site at position -31 (Ueno-Nishio et al., 1983). The fusion alters the

sequence of the -35 region to **TTGGCG**. An important feature of the altered sequence is the **TTG** triplet of the -35 region which is lacking in the original sequence. This alteration is apparently responsible for the 10-fold increase in the expression of glnL obtained with gene fusions (Ueno-Nishio et al., 1983). Conversely, a fusion at position -12 eliminated the -35 region and resulted in diminished ability to express the glnL gene.

NR_I binding sites. Both the glnF and glnG gene products are required for transcriptional activation of a number of Ntr operons. These include the Nif (nifLA) genes (Drummond et al., 1983; Merrick, 1983; Ow and Ausubel, 1983), hisJQHP which codes for components of the uptake system for histidine (Higgins and Ames, 1982; Ames and Nikaido, 1985) and the hut operon which codes for enzymes involved in the degradation of histidine (de Bruijn and Ausubel, 1981). Promoters of the glnALG operon are subject to both positive and negative control by the glnG product (NR_I). The Ntr promoter glnAp2 is subject to activation whereas the two minor promoters glnAp1 and glnLp are subject solely to repression.

Promoters subject to control by the glnLG gene products contain a common NR_I binding site. Dixon (1984) proposed a preliminary consensus repressor binding site for NR_I, on the basis of nucleotide sequence homology amoung Ntr-repressible promoters in enteric bacteria:

TGCACTANNNTGGGTGCAA

This sequence is similar to the NR_I binding consensus sequence proposed on the basis of DNA-binding studies (Ames and Nikaido, 1985):

AAAA

TGCACC TGGTGCA

TTTT

The consensus sequence has dyad symmetry and can accommodate a dimer of identical subunits in either a direct or an inverted repeat arrangement. Three dimensional modelling of a sequence in the glnA region of K. pneumoniae protected by NR_I (Hawkes et al., 1985), indicated that NR_I binds in a similar manner to other sequence-specific binding proteins (Pabo and Sauer, 1984).

Binding sites for NR_I in the glnALG promoter regions have been identified in vivo and in vitro (Ueno-Nishio et al., 1984; Ames and Nikaido, 1985; Reitzer and Magasanik 1986). Purified NR_I from S. typhimurium binds to five sites in the glnA promoter-regulatory region (Ames and Nikaido, 1985; Hirschman. 1985). The E. coli glnA promoter region, which is homologous to the corresponding region in S. typhimurium, has an identical number of binding sites for NR_I (Fig1.5) (Reitzer and Magasanik, 1986) with sequences that differ by no more than 2 bp at each binding site (Table 1.2). Site 1 overlaps the portion of the glnAp1 promoter homologous to the canonical **TTGACA** sequence and site 2 overlaps the transcriptional start site. The NR_I protein binds strongly to sites 1 and 2. in contrast sites 3, 4 and 5 are weak

Table 1.2. Comparison of NR_I binding sites in _E. coli_ and _S. typhimurium_.

Site	Bacterium	Nucleotide sequence
1	_S. typhimurium_	GCACCAATGTGGTGC
	E. coli	GCACCAACATGGTGC
2	_S. typhimurium_	GCACTATTTTGGTGC
	E. coli	GCACTATATTGGTGC
3	_S. typhimurium_	TCAC--CGTGGTGC
	E. coli	TCACATCGTGGTGC
4	_S. typhimurium_	GCACGATGGTGC
	E. coli	GCACGATGGTGC
5	_S. typhimurium_	ACGCCTTTTGGGGGC
	E. coli	ACGCCTTTTAGGGGC

binding sites localized between glnAp1 and glnAp2. The corresponding glnA promoter region in *K. pneumoniae* is somewhat different. Hawkes *et al*. (1985) demonstrated the occurrence of a NR_I binding site in a region presumably corresponding to glnAp1. This sequence is identical to the sequence at site 2 in *S. typhimurium*. Ames and Nikaido (1985) indicated the presence of a second NR_I binding site located upstream of the glnAp1 promoter. However, footprinting data obtained by Hawkes *et al*. (1985) indicated that there is only one major NR_I binding site in this region.

The NR_I protein has also been shown to bind to a region of DNA in the *E. coli* glnA-glnL intercistronic region (Fig.1.6) (Ueno-Nishio *et al*., 1984). The protected sequence (27 bp) overlaps the transcriptional start and the Pribnow sequence of the glnL promoter. Binding of NR_I to this site prevents the initiation of transcription by the σ^{70}-RNA polymerase holoenzyme (Reitzer and Magasanik, 1983) and a point mutation in this region abolishes NR_I repression of glnLp (Ueno-Nishio *et al*., 1984). A sequence identical to the NR_I binding site of *E. coli* glnLp is present in the *S. typhimurium* glnA-glnL intercistronic region (Hanau *et al*., 1983). Furthermore, the NR_I binding site of glnLp closely resembles a sequence overlapping the transcriptional start at glnAp1 (Reitzer and Magasanik, 1985). The homologous regions are shown below:

E. coli	glnAp1	**CATTGAAGCACTATATTGGTGCAACAT**
E. coli	glnLp	**CTATAATGCACTAAAATGGTGCAACCT**
S. typhimurium	glnLp	**CTATAATGCACTAAAATGGTGCAACCT**

1.2.6 Transcriptional regulation of the glnALG operon. The GS protein was initially assumed to be responsible for regulating its own synthesis and the synthesis of other proteins involved in nitrogen metabolism. These reports were based largely on the existence of mutants having a variety of phenotypes with mutations linked to and presumed to be in glnA. These mutants included glutamine auxotrophs, constitutive mutants which synthesized high levels of GS and histidase in the presence of ammonia and mutants which were Gln^{+} but produced low levels of GS under all conditions and failed to derepress histidase (GlnR or Reg^{-}) (Streicher et al., 1975; Streicher et al., 1976; Bender and Magasanik, 1977a; 1977b; Foor et al., 1978; Gillardin and Magasanik, 1978). The regulatory role of GS was questioned following the discovery of the glnL and glnG genes (McFarland et al., 1981). Fine structure deletion mapping and complementation analysis of the glnALG region in E. coli revealed that many of the mutations initially assumed to occur in the glnA gene were in fact present in the glnL or glnG genes.

Cells grown in nitrogen replete medium contain approximately five molecules of NR_I (Reitzer and Magasanik, 1983). When the nitrogen resources become limiting, a reduction in the glutamine/α-ketoglutarate ratio signals the NR_{II} catalyzed phosphorylation of NR_I (discussed in section 1.2.3). The

low level of NR_I-phosphate can fully activate the initiation of transcription at glnAp2 by σ^{60}-RNA polymerase. Initiation of transcription at glnAp2 elevates the levels of GS, NR_I and NR_{II}. Partial termination of transcription occurs at the Rho-independent terminator present in the glnA-glnL intercistronic region (Fig.1.6) (Ueno-Nishio et al., 1984; Rocha et al., 1985). This partially accounts for the approximately 14-fold higher levels of GS occurring in cells grown under conditions of limiting nitrogen (Reitzer and Magasanik, 1983; Ueno-Nishio et al., 1984). Activation of other Ntr operons occurs when the intracellular level of NR_I approaches 70 molecules per cell (Reitzer and Magasanik, 1983). The glnALG operon differs from other Ntr operons in that the low level of NR_I present in cells grown under conditions of nitrogen excess is sufficient to activate the transcription of glnALG, but not other Ntr operons, when the cells are shifted to a nitrogen deficient medium (Pahel et al., 1982).

The ability to mediate low-NR_I activation is due to the occurrence of the two high-affinity NR_I binding sites (sites 1 and 2) in the glnA control region. These sequences have been shown to exhibit enhancer-like properties (Reitzer and Magasanik, 1986). The two NR_I binding sites remain effective when moved 1400 bp upstream from glnAp2, or when placed in either orientation 2000 bp downstream from the glnA gene. Deletion of site 1 reduced low-NR_I activation and deletion of both sites 1 and 2 abolished low-NR_I mediated activation. The strong NR_I binding site at the

```
                                   -200
                                     |
CTG TAC TAC AGC GTC TAA GTG TTT TAG TTGCCGTGGAAACTTTTCGCCTGT

                         -200
                           |
CTCTGGCAGGCCTGGGATCGGTGGCAAGCACATCACGCCGGATCCGACGCAAATGCGTCT

              -150
                |
TATCCGGCCTACACGGTGATGATGTTGGTAGGCCGGAGCAGGTGAGTCGCTCTCCAACGT

-100                                                   -50
 |                                                      |
GAAGTTTGTCAGCTATCTGTAGCCCATCTCTGCATGGGCTTTTTTCTCCGTCAATTCTCT

                                                 +1
    -35                              -10          ---->
GATGC[TTCGCG]CTTTTTATCCGTAAAAAGC[TATAAT]GCACTAAAATGGTGCAACCTGTTC
                                 ///////////////////////////

AGGAGACTGCTTT ATG GCA ACA GGC ACG CAG CCC GAT GCT GGG CAG
```

Fig. 1.6. Nucleotide sequence of the <u>E. coli</u> <u>glnA-glnL</u> intercistronic region. The sequence is as reported by Ueno-Nishio <u>et al</u>.(1984). The coordinate +1 is defined as the transcriptional start. The -10 and -35 regions of the <u>glnL</u> promoter are boxed. The hatched bar indicates the location of the NR_I binding site. Bold print indicates the nucleotide sequence of the Rho-independent terminator.

glnL promoter was able to partially replace NR_I binding sites 1 and 2 when moved to the approximate location of site 2 but not from its normal position in the glnA-glnL intercistronic region. However, moving sites 1 and 2 closer to the start of transcription diminished initiation of transcription at glnAp2 (Reitzer and Magasanik, 1986)

Under these conditions, glnAp1 and glnLp are repressed by simple obstruction since both promoters are overlapped by strong NR_I binding sites. Readdition of ammonia to nitrogen starved cells results in an increase in the glutamine/α-ketoglutarate ratio and a reversal of events. The NR_I protein is inactivated by dephosphorylation and the intracellular level is gradually diluted by subsequent cell growth (Reitzer and Magasanik, 1983; 1985). Transcription of glnA proceeds from the glnAp1 promoter while transcriptional activity of the glnLp promoter maintains a low intracellular level of NR_I

1.2.7 Relationship between GlnG and NifA. The diazotroph K. pneumoniae differs from other enteric bacteria in its ability to fix nitrogen. In this species, a complex regulatory circuit involving a Nif-specific and a general Ntr system has been established (reviewed by Ausubel, 1984; Dixon, 1984). At least 17 genes, arranged in 7 or 8 operons, are required for the fixation of dinitrogen. One of these operons, the nifLA operon, regulates expression of the other Nif operons in conjunction with the glnF gene product (Fig. 1.4). The expression of the nifLA operon is

specifically activated by the products of *glnF* and *glnG* genes (Merrick, 1983; Ow and Ausubel, 1983) in concert with other nitrogen assimilation pathways. The *nifA* gene product then activates the transcription of the other Nif operons. The *nifL* gene product represses the Nif operons in response to the presence of oxygen or ammonia (Dixon, 1984).

Several lines of evidence indicate that the *nifA* and *glnG* genes are structurally and functionally related. The products of *nifA* and *glnG* are both basic polypeptides with very similar M_r and isoelectric points (Merrick, 1983). Both require the GlnF protein for activation. The *nifA* and *glnG* activated promoters both share an atypical consensus sequence in the -27 to -11 region. The *nifA* and *glnG* genes both occur as downstream genes in a two gene regulatory operon. When constitutively expressed, *nifA* can activate many of the operons normally activated by *glnG* (Drummond *et al*., 1983; Merrick, 1983; Ow and Ausubel, 1983)

The are however, some important differences between *nifA* and *glnG*. Although *glnG* can activate some of the Nif promoters, it does so relatively weakly, except at the *nifLA* promoter (Drummond *et al*., 1983; Merrick, 1983; Ow and Ausubel, 1983; Buck *et al*., 1985). Only activation mediated by *nifA* is susceptible to repression by nifL. While the *nifA* product can substitute for the *glnG* product as a positive activator, it cannot substitute for the *glnG* product as a negative regulator (Alvarez-Morales, 1984). A motif situated more than 100 bp upstream from the transcriptional start sites of

the Nif operons is required for full activation by nifA but has no effect on activation by glnG (Buck et al., 1986). Finally, the consensus sequences for the binding site of the individual proteins differ considerably (Buck et al., 1986; Reitzer and Magasanik, 1986).

The nifA and glnG genes of K. pneumoniae have been sequenced and shown to have highly conserved central domains (Buikema et al., 1985; Drummond et al., 1986). With the exception of a 20 amino acid sequence at the carboxy terminus, the amino and carboxy termini of the two proteins showed no significant homology. The N-terminal of the GlnG protein is the apparent site of phosphorylation by NR_{II}. The structural differences between the N-termini of the nifA and glnG products is in accord with the observation that the nifA product need not be converted to an active form (phosphorylated) to stimulate activation of transcription. The conserved region of 20 amino acids in the C-terminus forms the characteristic helix-turn-helix motif present in many DNA binding proteins. The carboxy-terminus of the nifA and glnG proteins is apparently involved in the binding of these proteins to DNA. The fact that the C-termini of the two proteins differ significantly is in agreement with the differences in the consensus sequences of the two binding sites.

The common properties of nifA and glnG suggests that these two genes may have a common evolutionary origin. Merrick (1983) proposed that the nifLA genes evolved in concert with

the Nif promoters to function specifically in regulation of the Nif regulon, whereas the glnLG products are involved in global nitrogen control.

1.3 *V. alginolyticus* GS. Bodasing *et al.* (1984) purified the GS from *V. alginolyticus* and showed that it is regulated by adenylylation and subject to feedback inhibition by amino acids and AMP. GS activity was regulated by nitrogen catabolite repression by $(NH_4)_2SO_4$ and glutamine but other amino acids enhanced the levels of GS activity. GS was not subject to glucose, fructose, glycerol or maltose catabolite repression and these sugars markedly enhanced GS activity.

More recently, a *V. alginolyticus* glutamine requiring mutant (*gln-1*) was isolated (P. Brandt, unpublished results). The *gln-1* mutant required glutamine for normal growth but grew at a slower rate on glutamate supplemented medium. In addition, this mutant was pleiotropically altered in the regulation of collagenase, suggesting a common element of control for GS and collagenase expression. It was therefore of interest to investigate the molecular structure and regulation of GS in order to understand nitrogen assimilation and its regulation in this species.

To this end, a *V. alginolyticus* genomic library was established in the positive selection vector p*Eco*R251. The fragment of DNA harbouring the *glnA* region was isolated from the library and the expression and regulation of the cloned gene in *E. coli* was investigated. Deletion analysis and DNA

sequencing revealed the structural organisation of this region and the origin of a GS transcript was mapped. Evidence is provided to support the occurrence of a regulatory cascade in this bacterium, with components that complement the E. coli Ntr system

CHAPTER TWO

CLONING OF *V. alginolyticus* *glnA* REGION IN *E. coli*

CHAPTER TWO

CLONING OF V. alginolyticus glnA REGION IN E. coli

Summary. A partial Sau3A library of V. alginolyticus genomic DNA was established in E. coli HB101 by insertional inactivation of the EcoR1 gene of pEcoR251. A GS gene was identified by complementation of the glnA deletion in E. coli ET8051 glnA, glnL, glnG deletion strain. The glnA region of V. alginolyticus was cloned on a 5.7 kb insert in pRM210. The origin of the DNA gene was confirmed by hybridization. Western blot analysis confirmed the immunological relatedness between the GS cloned in E. coli and the GS from V. alginolyticus.

2.1 INTRODUCTION

The ability to recombine molecules in vitro has opened the way to a detailed molecular analysis of genetic material from a variety of organisms. Since the construction of the first hybrid DNA molecule (Jackson et al., 1972), a wide range of cloning vehicles have emerged. E. coli and its plasmids constitute the most versatile type of host-vector system for DNA cloning due to the extensive body of genetic, biochemical and physiological knowledge pertaining to the organism. It has become common practice to clone and manipulate DNA fragments in E. coli to study basic features

of molecular biology before studying expression of the gene in another organism.

E. coli plasmids such as Col E1 (Hershfield et al., 1974) and pMB1 (Bolivar et al., 1977) have been modified to give rise to a series of vectors adapted for cloning DNA fragments ranging from a few hundred bp to 9 kb in size. Cloning vehicles based on the E. coli bacteriophage lambda have been used extensively. The cloning capacity of the vectors varies considerably with respect to the size of the clonable fragments. The M13-mp derivatives isolated by Messing and his collaborators (Messing, 1981; 1983) have proved useful in cloning smaller DNA fragments suitable for sequencing while the cosmids (Collins and Hohn, 1978) with a maximum capacity for 40 kb inserts are often the vectors of choice for the preparation of genomic libraries. Burke et al. (1987) have recently reported the generation of yeast artificial chromosomes with YAC vectors. This could provide a general method of cloning exogenous DNA fragments of several hundred kilobase pairs. Rapidly advancing DNA technology has led to the construction of cloning vehicles for both Gram-negative and Gram-positive bacteria, fungi, yeasts, plant and animal cells (for a compilation of available cloning vectors see Pouwels et al., 1986). Specialized vectors designed for the production of fused polypeptides (Silhavy and Beckwith, 1985), extracellular secretion of cloned gene products (Chrayeb, 1984), detecting promoters (Duvall et al., 1984; Koop et al., 1987) and terminators (McKenny et al., 1981) have also been developed.

The isolation of single-copy genes from complex genomes involves the cloning of random fragments and screening the resulting recombinants for the genes of interest. Two strategies may be employed to prepare genomic libraries. The first approach involves the complete digestion of genomic DNA with a restriction endonuclease and insertion of the cleaved fragments into an appropriate vector. This method does not exclude the possibility of cloning the gene of interest in two or more fragments. Where the identification of particular recombinants depends upon the expression of the cloned gene, it is essential to clone a fragment harbouring the entire gene. An alternative method is to generate a partial-digest library. Random cleavage of total cellular DNA reduces the possibility of systematic exclusion of sequences from the cloned library. The number of clones needed to obtain a representative genomic library for a given organism may be calculated from the formula:

$$N = \frac{\ln(1-P)}{\ln(1-f)}$$

where P is the desired probability, f is the fractional proportion of the genome in a single recombinant and N is the required number of recombinants (Clarke and Carbon, 1976)

The work described in this chapter includes the construction of a partial _Sau_3A library of _V. alginolyticus_ genomic DNA in the positive selection vector p_Eco_R251 (M. Zabeau, Plant Genetic Systems, Belgium). The vector contains the _E. coli_ _Eco_R1 gene under the control of the lambda rightward

promoter, the ampicillin (Ap) resistance gene and the pBR322 origin of replication. It was derived from the pcI857 plasmids described by Zabeau and Stanley (1982). The *Eco*R1 gene product expressed at high levels by the lambda promoter on p*Eco*R251, is lethal unless insertionally inactivated or regulated by plasmid pcI857 which contains a temperature sensitive lambda repressor gene (Remant *et al*., 1983). The GS gene of *V. alginolyticus* was purified from the genomic library by complementation of the *glnA* deletion in *E. coli glnA*, *ntrB*, *ntrC* deletion mutants (Pahel and Tyler, 1979). The deletion mutant *E. coli* YMC11 has been used to clone the structural gene for GS from a variety of organisms (Backman *et al*., 1981; Fisher *et al*., 1981; Fisher *et al*., 1984; Riccardi *et al*., 1985). Restriction endonuclease mapping and characterization of the cloned *V. alginolyticus glnA* region is presented.

2.2 MATERIALS AND METHODS

2.2.1 Bacteria and plasmids. Bacterial strains and plasmids used are listed in Table 2.1. p*Eco*R251 was a gift from M. Zabeau, Plant Genetic Systems, Ghent, Belgium. The plasmid has a unique *Bgl*II endonuclease cloning site located in the *Eco*RI gene.

2.2.2 Media. All media used are listed in Appendix B. *V. alginolyticus* was grown in peptone medium (Welton and Woods 1973, 1975). The *E. coli* strains were grown in Luria medium (Miller, 1972) and glucose minimal medium (Miller, 1972) with either $(NH_4)_2SO_4$ (1g/l) or glutamine (20 mM) as the sole nitrogen source.

2.2.3 Preparation of DNA. Plasmid DNA was prepared by the alkali-lysis method of Ish-Horowicz and Burke (1981) as described in Appendix C. *V. alginolyticus* chromosomal DNA was isolated by a modification of the method of Marmur (1961). Overnight *V. alginolyticus* cultures (100 ml) in peptone medium were harvested by centrifugation. Cells were resuspended in 4 ml of sucrose buffer (0.73 M sucrose, 10 mM EDTA, 10 mM Tris-hydrochloride, pH 8.0) containing lysozyme (4 mg/ml) and incubated at 37^0C for 1 hour (h) with shaking. The cell suspension was then maintained at 4^0C for 5 min before adding 2 ml of 0.2 M EDTA (pH 8.0). Lysis was achieved by the addition of sodium dodecyl sulphate (SDS) buffer (2% (w/v) SDS, 10 mM EDTA, 10 mM Tris-HCl, pH 8.0)

Table 2.1. Bacterial strains and plasmids

Strain	Relevant genotype	Reference
E. coli		
HB101	*glnA*$^+$,*glnL*$^+$,*glnG*$^+$, *pro*$^-$, *leu*$^-$, Ap^s	Maniatis *et al.* (1982)
YMC10	*glnA*$^+$,*glnL*$^+$,*glnG*$^+$,Ap^s	Backman *et al.* (1981)
ET8051	*glnA*$^-$,*glnL*$^-$,*glnG*$^-$,Ap^s	Pahel and Tyler (1979)
V. alginolyticus	wild-type	Welton and Woods (1973; 1975)

Plasmid	Description	Reference or source
p*Eco*R251	Ap^r,*Eco*RI	M Zabeau, Plant Genetic Systems, Belgium
pcI857	Kan^r,P_R repressor (ts)	Remaut *et al.* (1983)
pRM210	*glnA*$^+$,Ap^r	Current study
pRM211	*glnA*$^+$,Ap^r	Current study
pRM212	*glnA*$^-$,Ap^r	Current study
pRM213	*glnA*$^-$,Ap^r	Current study

and heating at $65^{0}C$ for 10 min. DNA in the cleared lysate was purified by centrifugation in cesium chloride-ethidium bromide (CsCl-EtBr) gradients as described in Appendix C.

2.2.4 Restriction enzyme digestion and electrophoresis of DNA. Standard techniques for the analysis of DNA were adopted from Maniatis et al. (1982). All restriction endonucleases were commercially produced and used in accordance with the specifications of the manufacturer (Appendix C).

2.2.5 Construction of a V. alginolyticus genomic library and the isolation of the V. alginolyticus glnA gene. The vector pEcoR251 was linearised with BglII endonuclease. V. alginolyticus DNA was partially digested with Sau3A endonuclease and fractionated on a sucrose density gradient (Appendix C). Fragments of genomic DNA (4-6 kb in size) were ligated with linearised vector in a 1:1 molar ratio. The ligation mixture was transformed into competent E. coli HB101 cells as described in Appendix C. Ampicillin resistant (Ap^r) transformants were pooled in lots of approximately 1000. Plasmid DNA extracted from each pool was used to transform E. coli ET8051. Complementation of the glnA deletion was selected for on minimal agar with $(NH_4)_2SO_4$ as the sole nitrogen source (Appendix B).

2.2.6 DNA hybridization. The techniques employed in this section of the work are described in Appendix C. Chromosomal DNA from V. alginolyticus was digested with

HindIII, EcoRI and KpnI endonucleases respectively. The restriction endonuclease digests were resolved by electrophoresis in 0.7% (w/v) agarose Tris-acetate gels and transferred to Gene Screen hybridization transfer membrane. An internal EcoR1 restriction fragment from the glnA region of V. alginolyticus was purified by elution onto DEAE-cellulose paper as described by Dretzen et al. (1981). The internal EcoR1 fragment was nick-translated (Rigby et al., 1977) using [α-^{32}P]-dCTP and used as a hybridization probe.

2.2.7 Immunoblot analysis. Details of the Western blotting technique are given in Appendix E. Purified V. alginolyticus GS and antibodies raised against this protein were provided by P.W. Brandt, Department of Microbiology, University of Cape Town, South Africa. E. coli YMC10, E. coli ET8051(pRM210) and E. coli ET8051(pRM211) were grown in glucose minimal medium. E. coli ET8051 and E. coli ET8051(pRM212) were grown in Luria medium (Appendix B). Proteins in crude cell extracts (Appendix D) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and transferred electrophoretically to nitrocellulose (Rybicki and von Wechmar, 1982). Resolved proteins were challenged with affinity purified (Rybicki, 1984) antibodies raised against V. alginolyticus GS (Bodasing et al., 1985).

2.3 RESULTS

2.3.1 Cloning of V. alginolyticus glnA gene in E. coli. A library of V. alginolyticus DNA was established in E. coli HB101 by insertional inactivation of the EcoR1 gene of pEcoR251. Competent E. coli HB101 cells were transformed with the ligation mixture described in section 2.2.5 and approximately 8000 Ap^r transformants were obtained. Plasmid DNA was extracted from a random selection of Ap^r colonies as described in Appendix C.1.1. All of the plasmids analyzed were found to contain insert DNA. The size of the insert DNA ranged from 4-12kb. Recombinants were pooled in lots of approximately 1000 and plasmid DNA was extracted from each pool and frozen (-70^{0}C) for long term storage. These pools of plasmid DNA were used to transform E. coli ET8051 glnA, glnL, glnG deletion strain. Twelve $glnA^+$ transformants were isolated and after restriction enzyme analysis, all plasmids extracted from the $glnA^+$ Ap^r colonies revealed identical PstI endonuclease restriction patterns. One recombinant plasmid designated pRM210 was selected for further study. Retransfer of this plasmid to E. coli ET8051 confirmed that it carried the glnA gene.

2.3.2 Reaction with GS antibodies. The presence of the V. alginolyticus glnA gene on pRM210 was confirmed by Western blotting. Crude protein extracts from E. coli YMC10, E. coli ET8051(pRM210) and purified V. alginolyticus GS were challenged with antibodies raised against V. alginolyticus GS. A protein which cross-reacted with V. alginolyticus GS

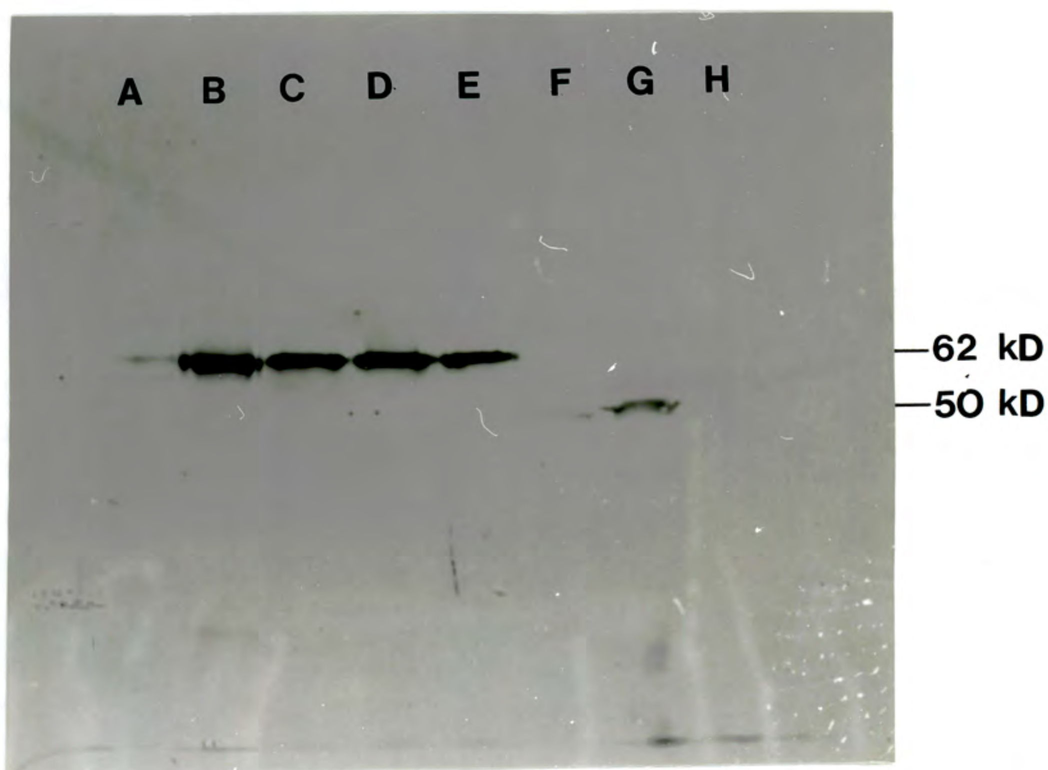


Fig. 2.1. Western blotting of GS from V. alginolyticus, E. coli YMC10 (wild-type) and E. coli ET8051 glnA$^-$, glnL$^-$, glnG$^-$ with and without pRM210. Proteins in crude cell extracts were resolved in 10% (w/v) SDS-PAGE gels and immunoblotted as described in Appendix E. Lane A: V. alginolyticus (75 μg protein); lane B: purified V. alginolyticus GS (25 μg protein); lanes C-E: E. coli ET8051(pRM210) (150, 200, 250 μg protein respectively); lane F: E. coli YMC10 (150 μg protein); lane G: E. coli YMC10 (250 μg protein); lane H: E. coli ET8051 (250 μg protein).

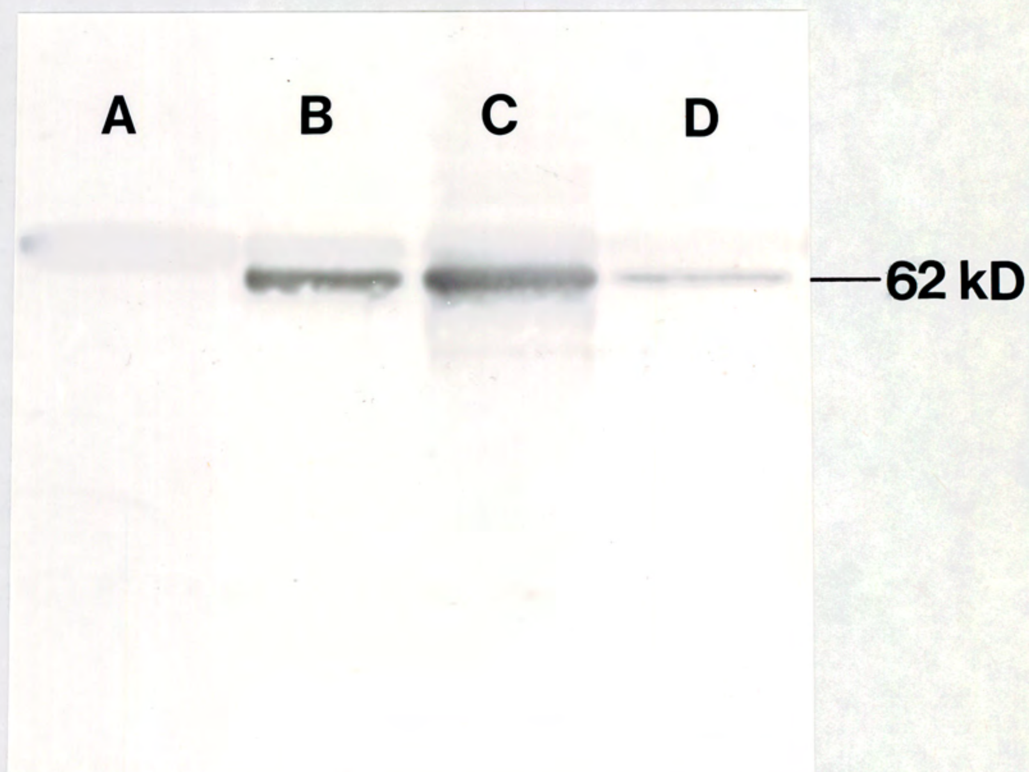


Fig. 2.2. Western blotting of GS from E. coli YMC11 glnA^{-}, glnL^{-}, glnG^{-} with and without pRM210, pRM211 or pRM212. Proteins (200 μg) in crude cell extracts were resolved in 10% (w/v) SDS-PAGE gels and immunoblotted as described in Appendix E. Lane A: E. coli ET8051; lane B: E. coli ET8051(pRM210); lane C: E. coli ET8051(pRM211); lane D: E. coli ET8051(pRM212).

antibody was produced in E. coli ET8051(pRM210) but not in E. coli ET8051 (Fig. 2.1). These bands corresponded with those of the purified V. alginolyticus GS. A faster migrating protein in the wild-type E. coli strain reacted with the antiserum to a lesser extent. The apparent M_r of the antigenic proteins was in good agreement with the apparent subunit M_r of V. alginolyticus and E. coli GS respectively (Stadtman and Ginsberg, 1974; Bodasing et al., 1985). Similar Western blotting experiments with cell extracts from E. coli ET8051(pRM211) and E. coli ET8051(pRM212) probed with the antiserum prepared against purified V. alginolyticus GS indicated that the GS monomer produced by pRM211 and pRM212 had the same M_r as the GS subunit produced by pRM210 (Fig. 2.2).

2.3.3 Restriction endonuclease mapping of the cloned V. alginolyticus DNA fragment. Single and double digestions with restriction endonucleases were employed to determine the locations of cleavage sites in pRM210 for a number of restriction enzymes (Fig. 2.3). Localization of the glnA region was achieved by the isolation and characterization of pRM210 deletion plasmids (Fig. 2.3). The V. alginolyticus glnA gene was located on a 2.4 kb fragment of insert DNA in pRM210.

2.3.4 DNA homology. The origin of the 5.7 kb insert in pRM210 was determined by Southern blotting and DNA hybridization (Fig. 2.4). The internal [α-^{32}P]-labelled EcoR1 and HindIII endonuclease digests hybridized with the

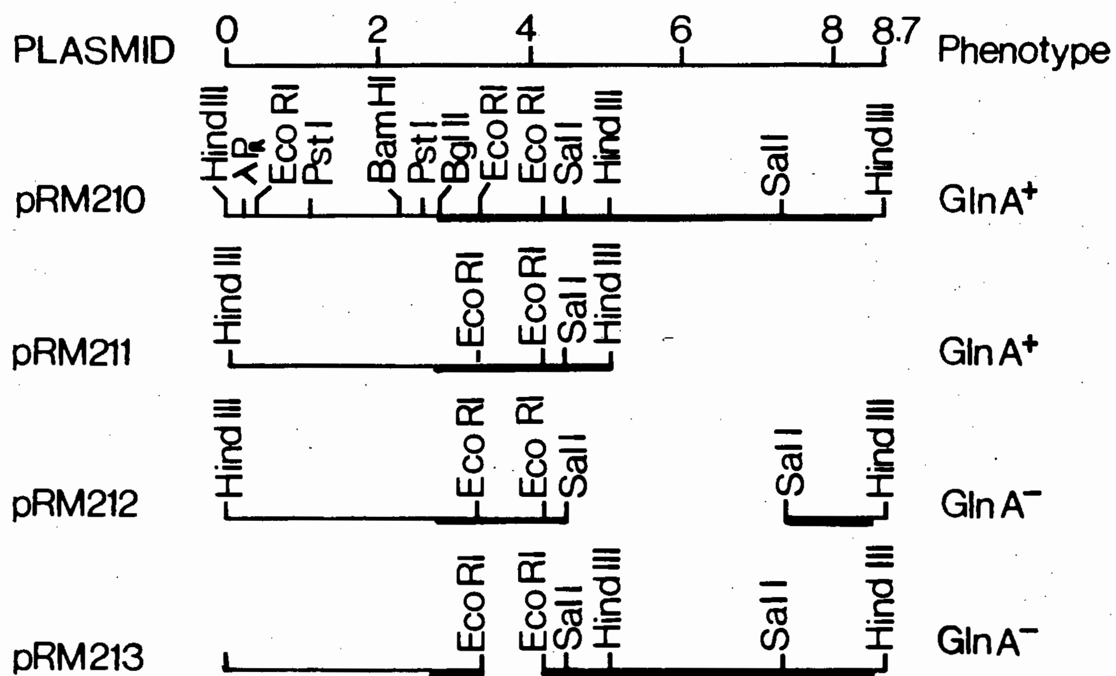


Fig. 2.3. Restriction and deletion map of pRM210

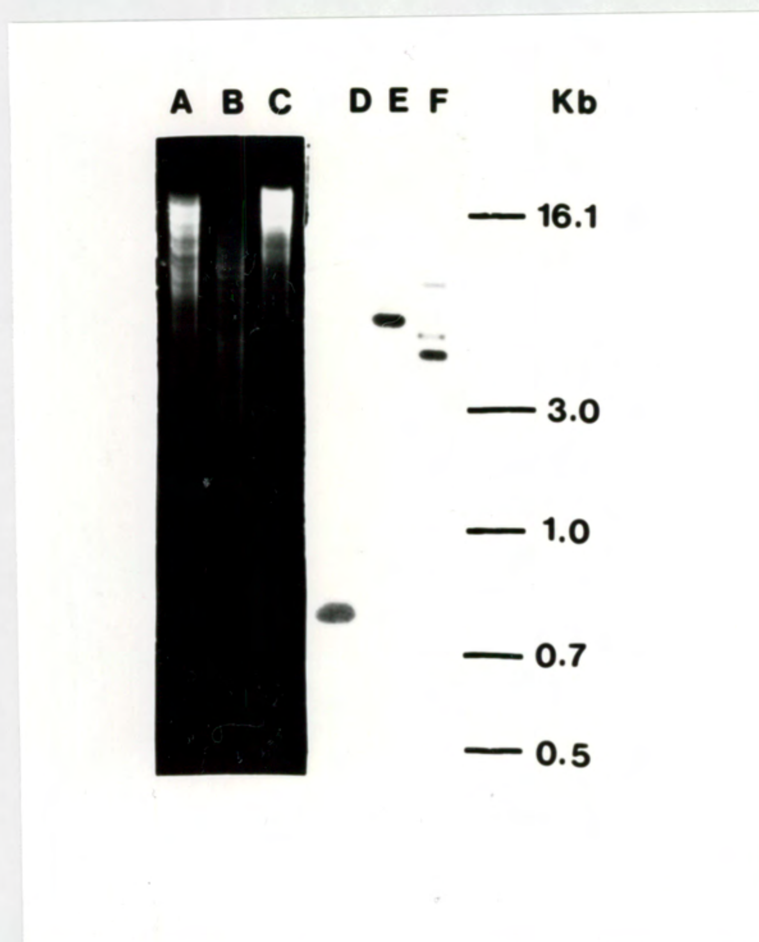


Fig. 2.4. Southern hybridization of an internal EcoRI restriction fragment of *V. alginolyticus* *glnA* gene with *V. alginolyticus* chromosomal DNA. Total cellular DNA was cleaved with the following restriction endonucleases and separated by agarose gel electrophoresis: Lane A: EcoRI; lane B: HindIII; lane C:KpnI; lanes D-F: autoradiograph of fragments of lanes A-C hybridized with [α-^{32}P]-labelled probe.

probe (Fig. 2.4, lanes D and E respectively). Lane F in Fig. 2.4 was a partial *KpnI* endonuclease digest and a number of hybridization bands were observed. The electrophoretic mobility of the homologous DNA sequence in the *EcoR1* endonuclease digest corresponded with that of the *EcoRI* restriction fragment of insert DNA.

2.4 DISCUSSION

The ability to isolate defined fragments of DNA facilitates the study of the structure and function of a gene and its relationship to other DNA sequences. To this end, a *V. alginolyticus* genomic library was established in the *E. coli* vector p*Eco*R251. The advantage of using a positive selection vector was demonstrated by the absence of parental plasmids in the Ap^r transformants examined. Statistically, for a 99% probability of isolating a given gene from an *E. coli* genomic library with an average insert size of 5 kb, 4000 recombinants would have to be obtained (Clarke and Carbon, 1976). The genome size of *V. alginolyticus* is as yet unknown but is expected to be in the same order of magnitude as that of *E. coli*. The number of Ap^r transformants (approximately 8000) obtained was therefore judged to be adequate for a good representation of the *V. alginolyticus* genome. The formulae used to calculate the number of clones required for full representation of all the DNA sequences in the insert DNA do however have their limitations. These formulae cannot be applied rigorously when constructing a gene bank with insert sizes covering a

wide range, due the differential kinetics of ligation of DNA fragments of different sizes. Libraries produced from restriction enzyme digests of insert DNA are likely to be lacking in DNA sequences located in regions in which there are fewer restriction endonuclease sites. Those sequences that are present in regions having numerous cleavage sites for the specific restriction enzyme have a greater probability of being cloned and occur in a higher frequency in the library.

The V. alginolyticus glnA gene was isolated from the genomic library and shown to function in E. coli ET8051. Although a number of Ap^r, $glnA^+$ transformants were analyzed, all of the recombinant plasmids harboured the same insert. This reflects one of the limitations of the cloning strategy employed, namely internal duplication of clones. A SDS-resistant, Ca^{2+} dependent serine exoprotease gene (Deane et al., 1987) and a sucrose utilization system (Scholle et al., 1987) have been purified from the same V. alginolyticus genomic library. The genes were cloned on 7.1 and 10.4 kb fragments respectively. Isolation of genes other than the glnA gene from the V. alginolyticus genomic library suggests that the library is a fair representation of the V. alginolyticus genome. The origin of the glnA gene was confirmed by DNA hybridization. Single fragments of V. alginolyticus chromosomal DNA from the EcoRI and HindIII endonuclease digests hybridized with the probe. This result was in agreement with the restriction map of pRM210.

The *V. alginolyticus* *glnA* gene was located on a 2.4 kb of insert DNA in pRM210. Interestingly, the deletion plasmid pRM212 which harboured a 1.9 kb insert, displayed a $GlnA^-$ phenotype but was capable of directing synthesis of a protein having the same M_r mass as the GS subunit produced by pRM210 and *V. alginolyticus*. This suggested that the deletion plasmid pRM212 had the carboxyterminus of the *glnA* gene deleted. In *E. coli* the structural gene for GS is part of the complex *glnALG* operon (Pahel *et al*., 1982). The operon is located on a 4.5 kb fragment of DNA and is transcribed counterclockwise from *glnA* to *glnG* (Backman *et al*., 1981; MacNeil *et al*., 1982). The *glnA* region of *V. alginolyticus* was cloned on a 5.7 kb fragment of DNA. Deletion of a 3.27 kb fragment of insert DNA from pRM210 resulted in the deletion plasmid pRM211, which displayed a $GlnA^+$ phenotype. The 5.7 kb fragment of *V. alginolyticus* genomic DNA was therefore considered large enough to accommodate a putative *glnALG* operon.

When the entire *glnA* gene was present on a plasmid, it was capable of directing the synthesis of GS in *E. coli* *glnA* deletion mutants as judged by Western blotting. Immunoblot analysis revealed some degree of protein homology between *V. alginolyticus* and *E. coli* *glnA* products. The apparent M_r of the antigens was in good agreement with the apparent subunit M_r of *V. alginolyticus* and *E. coli* GS respectively (Stadtman and Ginsberg, 1974; Bodasing *et al*., 1985). Antibodies raised against *V. alginolyticus* GS have also been shown to cross react with the GS of the Gram-negative

autotrophic chemolithotroph *Thiobacillus ferrooxidans* (Barros *et al*., 1986). These observations extend the findings that GS from heterotrophic Gram-negative bacteria show varying degrees of homology (Tronick *et al*., 1973).

CHAPTER THREE

EXPRESSION AND REGULATION OF THE CLONED *V. alginolyticus* GS GENE IN *E. coli*

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Summary. GS synthesis in *V. alginolyticus* was regulated by temperature, oxygen and nitrogen availability. The cloned *V. alginolyticus* *glnA* gene was expressed in *E. coli* from a promoter contained within the cloned fragment. *V. alginolyticus* *glnA* expression in *E. coli* from pRM210 was subject to regulation by temperature, oxygen and nitrogen levels. GS specific activity in an *E. coli* wild-type strain was not affected by temperature or oxygen. The expression of GS on pRM211, a deletion derivative of pRM210, was not regulated by temperature, oxygen or nitrogen levels in *E. coli*. Plasmids pRM210 and pRM211 enabled an *E. coli* *glnA*, *glnL*, *glnG* deletion mutant to utilize arginine or low levels of glutamine as nitrogen sources. In contrast, transactivation of histidase production in the *E. coli* *glnA*, *glnL*, *glnG* strain containing the *K. aerogenes* *hut* operon did not occur. *E. coli* *glnF* deletion strains containing pRM210 or pRM211 were unable to grow in minimal medium containing arginine as the sole nitrogen source. The cloned *V. alginolyticus* DNA fragment was able to activate expression of the cloned *E. coli* *glnA* gene *in trans*, under conditions of limiting nitrogen. The *E. coli* *glnL*, *glnG* region cross hybridized with a fragment of DNA located downstream of the *glnA* structural gene in pRM210. It is concluded that the *V. alginolyticus* *glnA* gene is regulated by genes analogous to

the glnL, glnG genes of E. coli, whose products participate in the regulatory cascade system.

3.1 INTRODUCTION

Expression of GS in Gram-negative and Gram-positive bacteria is subject to regulation by nitrogen availability in the growth medium. In the enteric bacteria, as well as in most Gram-negative species, GS activity can be regulated by covalent modification through an adenylylation-deadenylylation system. Transcriptional regulation of the glnA gene in the enteric bacteria is mediated by the concerted effort of the products of the glnL and glnG genes which are contiguous with the glnA structural gene, as well as by the product of the unlinked glnF gene. Regulation of GS in the Gram-negative bacteria E. coli, K. pneumoniae and S. typhimurium has been discussed fully in the general introduction.

The Ntr genes also regulate the expression of numerous genes involved in active transport and utilization of amino acids, and in nitrogen fixation (Kustu et al., 1979; Magasanik, 1982; Drummond et al., 1983; Ames and Nikaido, 1985; Merrick and Stewart, 1985). The histidine (hut) and proline (put) utilization operons are Ntr in Klebsiella species. However, there is no evidence that the hut and put operons in S. typhimurium are Ntr (Magasanik and Neidhardt, 1987). Bender et al. (1983) have shown that in K. aerogenes mutations in the nac gene prevented nitrogen regulation of hut and put

but not of *glnA*. On the basis of these findings, Magasanik and Neidhardt (1987) proposed that the expression of the *nac* gene is Ntr and that this gene activates the *hut* and *put* operons. The *nac* gene has hitherto not been identified in *E. coli*.

Although the Ntr system has been demonstrated in the Gram-negative enterobacteria there is no evidence for regulatory Ntr genes in the Gram-positive bacteria studied to date (Fisher and Sonenshein, 1984; Schreier *et al*., 1985; Usdin *et al*., 1986; Janssen *et al*., 1987). Ammonia-generating catabolic enzymes of the Gram-positive *Bacillus subtilis* species are not activated in response to nitrogen limitation and GS synthesis is derepressed only when cells are under severe nitrogen limitation (Deuel *et al*., 1970; Schreier *et al*., 1982; Fischer and Sonenshein, 1984). GS from *B. subtilis* is regulated by divalent cations and by feedback inhibition. However, there is no evidence for an adenylylation system in *B. subtilis*, nor is the *E. coli* adenylylating system able to modify the *B. subtilis* enzyme *in vitro* (Deuel *et al*., 1970; Deuel and Stadtman, 1970; Deuel and Prusiner, 1974). Schreier and co-workers (1982) have shown that the cloned *B. subtilis* GS gene is autoregulated and is not dependent on the *E. coli* Ntr system. Studies employing *lacZ* fusions indicate that the GS subunit can act as a negative regulator of expression from the *glnA* promoter of *B. subtilis*. The isolation of a class of glutamine requiring mutants of *B. subtilis* which are pleiotropically altered in the regulation of carbohydrate

metabolism and the expression of the hut operon suggests that the GS protein and/or its enzymatic product is involved in the regulation of cellular metabolism in this bacterium (Fischer and Sonenshein, 1977; 1984).

Strauch et al. (1988) have recently determined the nucleotide sequence of the B. subtilis glnA region. These workers identified an open reading frame (ORF) preceding the glnA gene, which apparently forms part of an operon containing the glnA gene. The product of the upstream gene has been proposed to play a role in the regulation of expression of GS since its deduced amino acid sequence is similar to that of certain repressors, and mutations in this ORF lead to constitutive transcription of both this gene and glnA (Schreier et al., manuscript in preparation). Located downstream of the glnA gene are two ORFs, each of which is preceded by a consensus ribosome binding sequence and capable of encoding a small hydrophobic polypeptide. The larger downstream ORF is preceded by a consensus sequence for interaction with the major vegetative ($E\sigma^{43}$) form of RNA polymerase. The functional significance of the downstream ORFs remains to be established since the DNA downstream from glnA is known not to be involved in the regulation of the amount of GS synthesized under conditions of nitrogen limitation.

The derived amino acid sequence of B. subtilis GS closely resembles that of the Clostridium acetobutylicum GS (Strauch et al., 1988). C. acetobutylicum is a Gram-positive spore

forming obligate anaerobe. The regulation of GS in *C. acetobutylicum* parallels that of *B. subtilis* in that the expression of *glnA* is regulated by nitrogen availability in the growth medium but the enzyme is not adenylylated (Usdin *et al*., 1986). However, the regions downstream of the *glnA* gene in these two bacteria differ significantly. The *glnA* region of *C. acetobutylicum* contains a downstream region with an extensive stretch of inverted repeat sequences and a sequence with promoter activity which is orientated towards the *glnA* gene (Janssen *et al*., 1987). The downstream putative promoter and the inverted repeat region enhance the production of GS under conditions of limiting nitrogen but are apparently not involved in the repression of GS levels in cells grown under conditions of nitrogen excess.

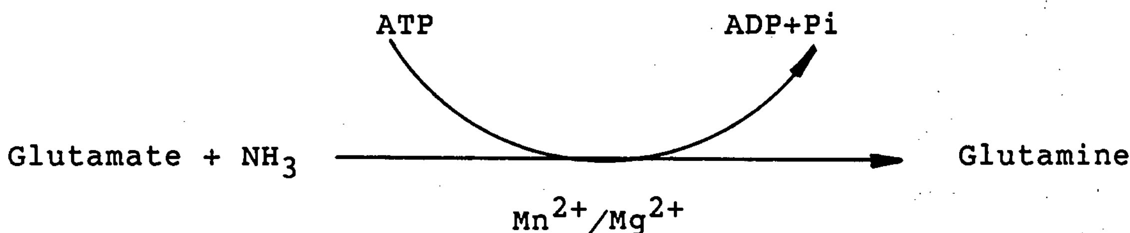
The GS subunit of *V. alginolyticus* has been purified and shown to be regulated by an adenylylation-deadenylylation system which is typical of all Gram-negative bacteria (Bodasing *et al*., 1985). Some aspects of nitrogen regulation in *V. alginolyticus* do however parallel *B. subtilis* rather than the more closely related Gram-negative bacteria. In *V. alginolyticus* and *B. subtilis*, true extracellular proteases are produced during the stationary growth phase (Welton and Woods, 1973; Welton and Woods, 1975; Priest, 1977; Long *et al*., 1981); protease production is rifampicin-insensitive (Both *et al*., 1972; O'Connor *et al*., 1978; Reid *et al*., 1978) and is subject to end-product and catabolite repression which is not relieved by cyclic AMP (Priest, 1977; Long *et al*., 1981; Glenn, 1976; Reid *et

al., 1978). In addition, histidine rather than urocanic acid is the inducer of the hut operons of V. alginolyticus and B. subtilis.

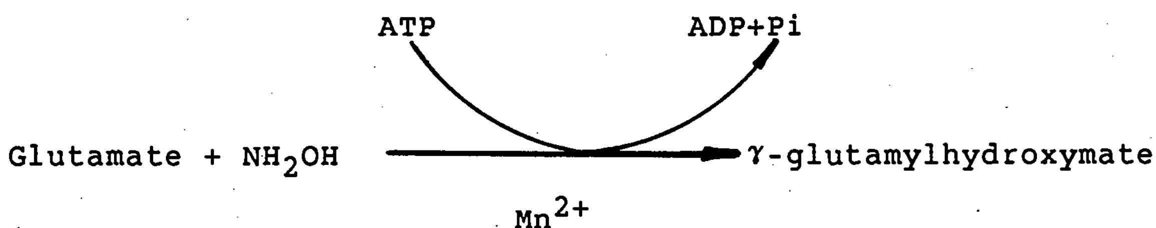
The work described in this chapter examines the expression of the cloned V. alginolyticus GS gene in E. coli and was directed towards establishing whether the V. alginolyticus glnA region contained genes which are functionally analogous to the Ntr genes of the enteric bacteria.

A variety of enzymic procedures have been employed for the measurement of GS activity in E. coli (Stadtman et al., 1979). These methods derive from the ability of the bacterial GS to catalyze a number of enzymatic reactions (Stadtman and Ginsburg, 1974):

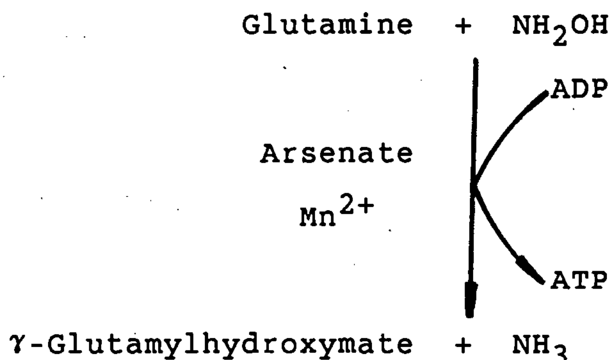
I Biosynthetic reaction



II Synthetic or forward transferase reaction



III γ-Glutamyl transferase reaction



The biosynthetic assay is suitable for estimating the degree of adenylylation (Mn^{2+} dependent activity) or deadenylylation (Mg^{2+} dependent activity) of GS while the forward transferase assay detects only the deadenylylated (active) form of the enzyme.

The γ-glutamyl transferase (γ-GT) assay of Shapiro and Stadtman (1970) enables the measurement of total GS activity and has been used most extensively. This assay is useful

during the purification of GS, or in studying activities in cell extracts, as it is less subject to interference by contaminating enzymes in comparison with the biosynthetic and synthetic assays. The γ-glutamyl transferase activity of GS has as yet no known physiological function (Wohlhueter *et al.*, 1973).

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains, plasmids and phages. The bacterial strains are listed in Table 3.1. Plasmids and phages used are listed in Table 3.2. *V. alginolyticus* was grown in minimal medium (Long *et al.*, 1981) containing glutamate (15 mM) and glutamine (0.15-15 mM). The *E. coli* strains were grown in Luria medium (Miller, 1972) and glucose minimal medium (Miller, 1972) supplemented with either $(NH_4)_2SO_4$ (20 mM), glutamine (0.15-20 mM), glutamate (15 mM) or arginine (10 mM) as the nitrogen source.

3.2.2 Enzyme assays. The colorimetric assays employed are described in Appendix D. GS activity in *E. coli* strains was determined in hexadecyltrimethyl ammonium bromide (CTAB) permeabalized cells (0.1 mg/ml) employing the γ-glutamyl transferase (γ-GT) assay of Shapiro and Stadtman (1970). GS activity in *V. alginolyticus* was determined in sonicated extracts as described by Bodasing *et al.* (1985). Histidase activity was determined in toluene permeabilized cells (25% (v/v)) employing a modification of the methods of Hartwell and Magasanik (1963) and Chasin and Magasanik (1968).

Table 3.1. Bacterial strains

Strain	Relevant genotype	Reference or source
E. coli		
YMC10	*glnA*$^+$,*glnL*$^+$,*glnG*$^+$	Backman *et al*. (1981)
YMC11	*glnA*$^-$,*glnL*$^-$,*glnG*$^-$	Backman *et al*. (1981)
YMC22	*glnA*$^+$,*glnF*$^-$	B. Magasanik (Massachusetts Inst. of Technology)
JM105	*thi*,*rpsL*,*endA*, *sbcB15*,*hspR4*, (lac-pro AB), [F,*tra* 36,proAB, *lacI*^{q} Z M15	Yanisch-Perron *et al*. (1985)

Table 3.2. Plasmids and phages

Plasmid	Description	Reference
pcI857	P_R repressor(ts)Kan^r	Remaut *et al*. (1983)
p804	*glnL*$^+$,*glnG*$^+$,Ap^r	Tuli *et al*. (1982)
pgln6	*glnA*$^+$,Ap^r,Tc^r	Backman *et al*. (1981)
pEGS	*glnA*$^+$,Cm^r	Current study
pACYC184	Cm^r,Tc^r	Chang and Cohen (1978)
pUC18	Ap^r	Messing and Vieira (1982)
pRM210	*glnA*$^+$,Ap^r	Current Study
pRM211	*glnA*$^+$,Ap^r	Current Study
pRM213	*glnA*$^-$,Ap^r	Current Study
pVNTR1	*glnA*$^-$,Ap^r	Current Study
pVNTR2	*glnA*$^-$,Ap^r	Current Study

Bacteriophage	Description	Reference
M13-mp18	Ap^r	Messing and Vieira (1982)
λENTR	Ap^r,*glnL*-,*glnG*$^-$	Current Study

Protein estimations were by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

3.2.3 Serological analysis. Enzyme-linked immunosorbent assays (ELISAs) (Clark and Adams, 1977) were carried out for the serological analysis of GS produced in E. coli strains (Appendix E). Antigenic determinants in sonicated extracts (Appendix D) were reacted with affinity purified antibodies (Rybicki, 1984) raised against V. alginolyticus GS (Bodasing et al., 1985)

3.2.4 Construction of plasmids and phages. Standard techniques for manipulation of DNA (Maniatis et al., 1982) were employed (Appendix C). Restriction maps of cloning vectors are given in Appendix F. Plasmids pRM210, pRM211 and pRM213 have been described in Chapter 2. The plasmids pVNTR1 and pVNTR2 are subclones of pRM210 constructed in E. coli JM105 by insertional inactivation of the γ-galactosidase gene in pUC18 plasmid. pVNTR1 was constructed by ligating a 3.7 kb HindIII endonuclease fragment from pRM210 with HindIII endonuclease linearised pUC18. pVNTR2 resulted from the insertion of a 2.75 kb SalI restriction fragment of V. alginolyticus DNA from pRM210 into the SalI restriction site of pUC18. Ligation mixes were used to transform competent E. coli JM105 cells and lac^- Ap^r transformants were selected on Luria agar (Appendix B) supplemented with 100 ug/ml Ap, 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 0.004% (w/v) 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal). The orientation of the

insert DNA was determined by restriction analysis of plasmid DNA isolated from *lac*$^-$ Ap^r transformants (Fig 3.1). pEGS is a Col E1 compatible derivative of pgln6 (Fig 3.2). A 2.8 kb *Cla*I restriction fragment of *E. coli* specific DNA from pgln6 was cloned into the *Bam*HI restriction site of the vector pACYC184. The ligation mixture was used to transform competent *E. coli* YMCII cells and Cm^r, Ap^s, Tc^s, *glnA*$^+$ recombinants were selected and verified by restriction analysis. The phage λENTR is a subclone of p804. A *Pvu*II endonuclease fragment spanning the carboxy terminus of the *glnL* gene and the amino terminus of the *glnG* gene of *E. coli* was cloned into the *Sma*I restriction site of the filamentous phage M13-mp18 (Fig 3.2). The ligation mixture of plasmid and M13 RF DNA was used to transform competent *E. coli* JM105 cells and *lac*$^-$ plaques selected on H agar (Appendix B) supplemented with 0.2 mM IPTG and 0.004% (w/v) Xgal. Restriction analysis of M13 RF DNA isolated from *lac*$^-$ transformants confirmed the presence of insert DNA.

3.2.5 DNA hybridization. Southern blotting and DNA hybridization techniques are described in Appendix C. Plasmid DNA isolated from *E. coli* YMCII(pRM210) and *E. coli* YMCII(pRM211) was digested with *Hind*III and *Sal*I endonucleases. RF DNA isolated from *E. coli* JM105(λENTR) was digested with *Pvu*II endonuclease. The double and single restriction endonuclease digests were resolved by electrophoresis in a 1% (w/v) agarose Tris-acetate gel and transferred to Gene Screen hybridization transfer membrane. M13 RF DNA isolated from *E. coli* JM105(λENTR) was nick-

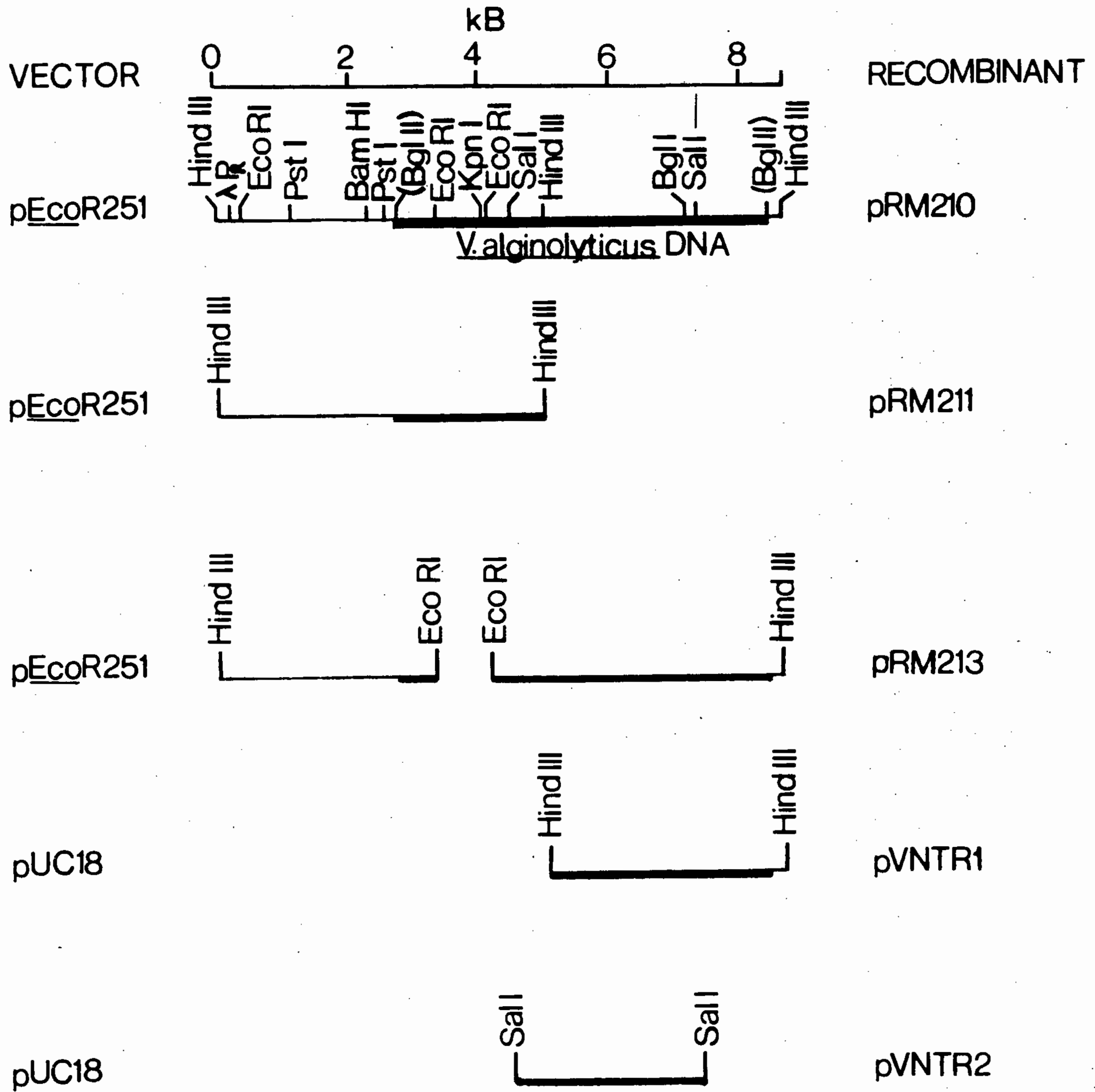


Fig. 3.1. Restriction map, deletions and sub-clones of pRM210.

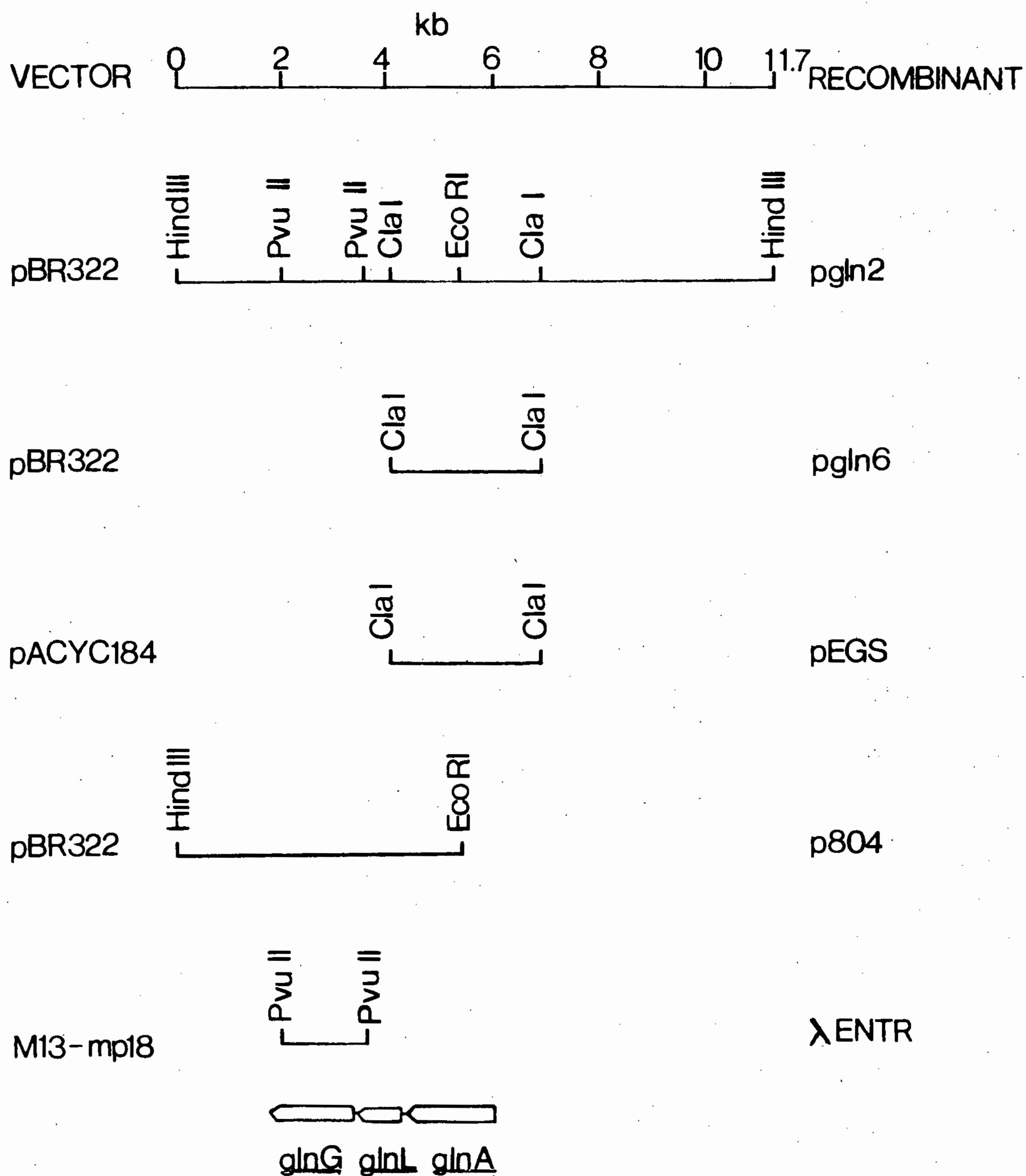


Fig 3.2. Regions of E. coli gln operon DNA on various vectors.

translated (Rigby *et al.*, 1977) using [α-^{32}P]-dCTP (3000 Ci/mmol) obtained from Amersham, and was used as a hybridization probe.

3.3 RESULTS

3.3.1 GS activity. GS activity in *V. alginolyticus* was regulated by nitrogen levels (Table 3.3). This observation was in agreement with the findings of Bodasing *et al.* (1985). The expression of GS activity in *V. alginolyticus* cells grown aerobically at 30^{0}C was induced approximately 5-fold under conditions of limiting nitrogen (0.15 mM glutamine) and was repressed by excess nitrogen (15 mM glutamine). GS specific activity in *V. alginolyticus* was also affected by temperature and oxygen (Table 3.4). Approximately 5-fold more GS activity was obtained in aerated *V. alginolyticus* cells at 30^{0}C than in standing cultures at 30^{0}C or aerated cultures at 37^{0}C. The effect of temperature and oxygen (Table 3.4) on the expression of GS activity in *E. coli* YMC10 (wild-type) and *E. coli* YMC11 *glnA*, *glnL*, *glnG* deletion mutant containing pRM210 or pRM211, was determined in minimal medium containing 15 mM glutamate and 0.15 mM glutamine as nitrogen sources. The GS specific activity of *E. coli* YMC10 wild-type cells was not affected by either temperature or oxygen. However, the expression of GS activity of *E. coli* YMC11(pRM210) was markedly affected by temperature and oxygen. At 30^{0}C approximately 5-fold higher expression of GS activity was obtained than at 37^{0}C. Under conditions of low oxygen very

Table 3.3. Effect of nitrogen levels on the expression of GS activity in V. alginolyticus, E. coli YMC10 (wild-type) and E. coli YMC11 glnA$^-$, glnL$^-$, glnG$^-$ containing pRM210 or pRM211.

Strain	GS activity	
	0.15 mM glutamine	15.00 mM glutamine
V. alginolyticus	0.50	0.11
E. coli YMC10	1.08	0.28
E. coli YMC11(pRM210)	1.15	0.24
E. coli YMC11(pRM211)	0.007	0.007
E. coli YMC11	0	0

Overnight cultures in minimal medium containing 15 mM glutamate and 0.15 mM glutamine were diluted into fresh medium containing 15 mM glutamate and 0.15 or 15 mM glutamine. The cells were grown aerobically at 30°C for 2-3 generations before harvesting. GS activity is expressed as μmol γGT produced/min/mg protein.

Table 3.4. Effect of temperature and oxygen on the expression of GS activity in V. alginolyticus, E. coli YMC10 (wild-type) and E. coli YMC11 glnA⁻, glnL⁻, glnG⁻ containing pcI857 and/or pRM210 or pRM211. Overnight cultures in minimal medium, containing 15 mM glutamate and 0.15 mM glutamine, were diluted into fresh medium and the cells grown for 2-3 generations before harvesting.

Strain	GS activity[a]			
	Temperature[b]		Oxygen[c]	
	30^0C	37^0C	+	-
V. alginolyticus	0.50	0.06	0.50	0.10
E. coli YMC10	1.08	1.05	1.08	1.07
E. coli YMC11				
(pRM210)	1.15	0.20	1.15	0.14
E. coli YMC11				
(pRM210,pcI857)	1.08	0.21	1.06	0.13
E. coli YMC11				
(pRM211)	0.007	0.008	0.006	0.006
E. coli YMC11				
(pRM211, pcI857)	0.006	0.007	0.006	0.007
E. coli YMC11	0	0	0	0

[a] GS activity is expressed as umol γ-glutamyl hydroxymate formed/min/mg protein

[b] Cultures were grown at 30 or 37^0C with aeration by shaking.

[c] Cultures were aerated (+oxygen) by shaking 100 ml of medium in 500 ml flasks fitted with loose fitting aluminium caps. Unaerated cultures (-oxygen) were incubated standing. Growth was at 30^0C.

low levels of GS activity were produced by E. coli YMC11(pRM210). These very low levels of GS activity were produced by E. coli YMC11(pRM211) cells grown at 30 or 37^{0}C and with or without oxygen. The low levels of GS activity were correlated with slower growth rates of the cultures. The mean doubling time for E. coli YMC11 with pRM210 or pRM211, grown under conditions of limiting nitrogen (0.15 mM glutamine) was 150 and 190 min respectively. GS activity was not detected in crude extracts of E. coli YMC11.

The effects of temperature and oxygen were also investigated in E. coli YMC11(pRM210, pcI857) and E. coli YMC11(pRM211, pcI857) (Table 3.4). The presence of the temperature sensitive lambda repressor gene on pcI857 did not affect the expression of GS activity on pRM210 or pRM211 under the various experimental conditions.

The effect of nitrogen levels on GS activity was investigated in E. coli YMC10 and E. coli YMC11 containing pRM210 or pRM211 (Table 3.3). GS specific activity in aerated cultures of E. coli YMC10 wild-type cells at 30^{0}C was induced approximately 4-fold in nitrogen limiting medium and repressed by excess glutamine. GS activity in E. coli YMC11(pRM210) was induced 5-fold under conditions of limiting nitrogen and was repressed by excess nitrogen. The very low levels of GS activity observed in E. coli YMC11(pRM211) cells were not affected by nitrogen levels.

3.3.2 Heat inactivation of the cloned *V. alginolyticus* GS. The rate of inactivation of *V. alginolyticus* GS in *E. coli* extracts was investigated. Protein extracts of *E. coli* YMC11(pRM210) were maintained at 30 and 37^{0}C and assayed for GS activity at timed intervals, over an eight hour period. The rates of inactivation of the cloned GS enzyme at 30 and 37^{0}C were similar (Fig 3.3).

3.3.3 Serological analysis of GS expression. The effect of temperature and oxygen on the expression of *V. alginolyticus* GS in *E. coli* was further investigated employing the ELISA technique. *E. coli* YMC11(pRM210) was grown in minimal medium containing 15 mM glutamate and 0.15 mM glutamine as nitrogen sources. Proteins in sonicated cell extracts were reacted with antibodies raised against purified *V. alginolyticus* GS. Serial dilutions of cell extracts in the range 0.02-200 μg/ml protein were analyzed. At a protein concentration of 200 μg/ml the amount of cross-reacting material present in extracts from cells grown aerobically at 30^{0}C was 3-fold greater than that present in extracts from cells grown at 37^{0}C (Fig 3.4). At the same protein concentration (200 μg/ml), the amount of antigen present in extracts of cells grown at 30^{0}C under conditions of high oxygen was 4.5-fold higher than that of cells grown under conditions of low oxygen (Fig 3.5)

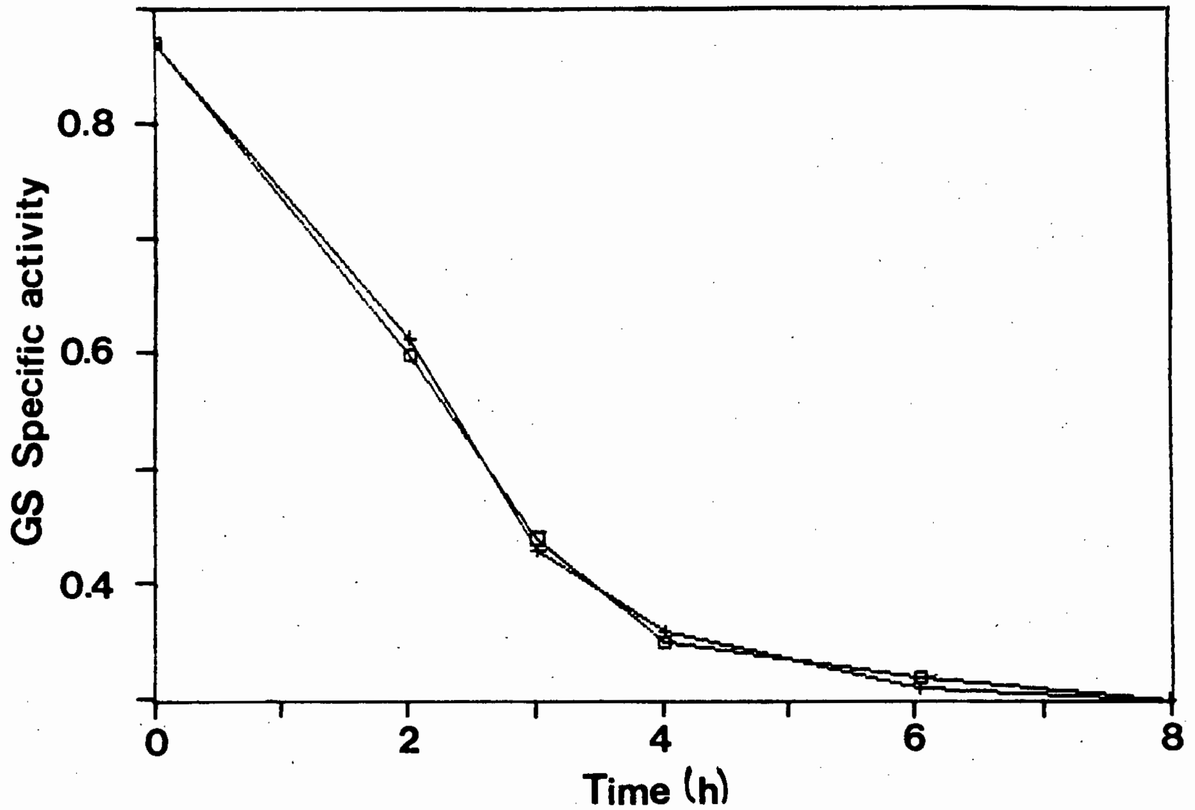


Fig. 3.3. Heat inactivation of the cloned V. alginolyticus GS. E. coli YMC11(pRM210) was grown in minimal medium containing 15 mM glutamate and 0.15 mM glutamine at 30°C with aeration. Sonicated cell extracts were then maintained at 30 (x) and 37°C (□) and assayed for GS activity at timed intervals. GS activity is expressed as μmol γGT produced/min/mg protein.

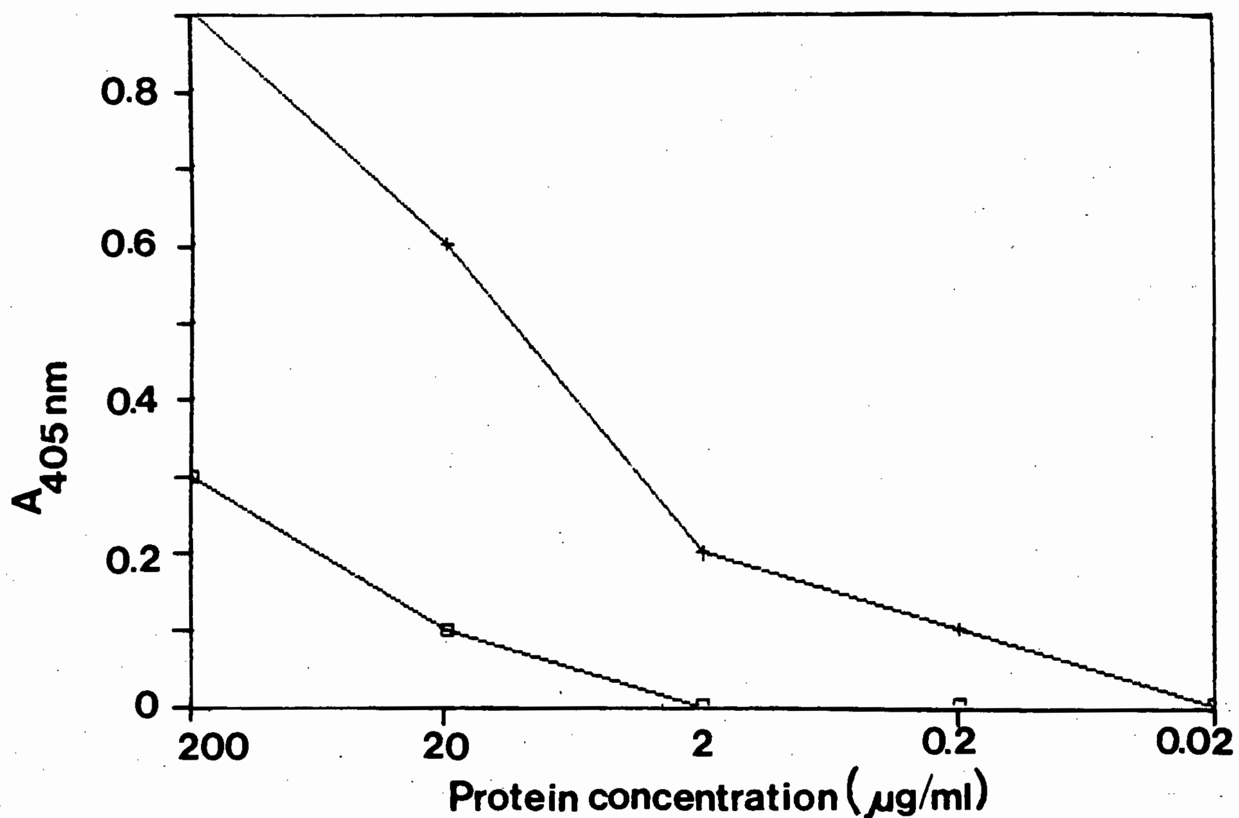


Fig. 3.4. ELISA absorbance values of V. alginolyticus GS produced at 30 (x) and 37^{0}C (□) in E. coli cells. Protein extracts from E. coli YMC11(pRM210), grown in minimal medium containing 15 mM glutamate and 0.15 mM glutamine, were reacted with antibodies raised against purified V. alginolyticus GS.

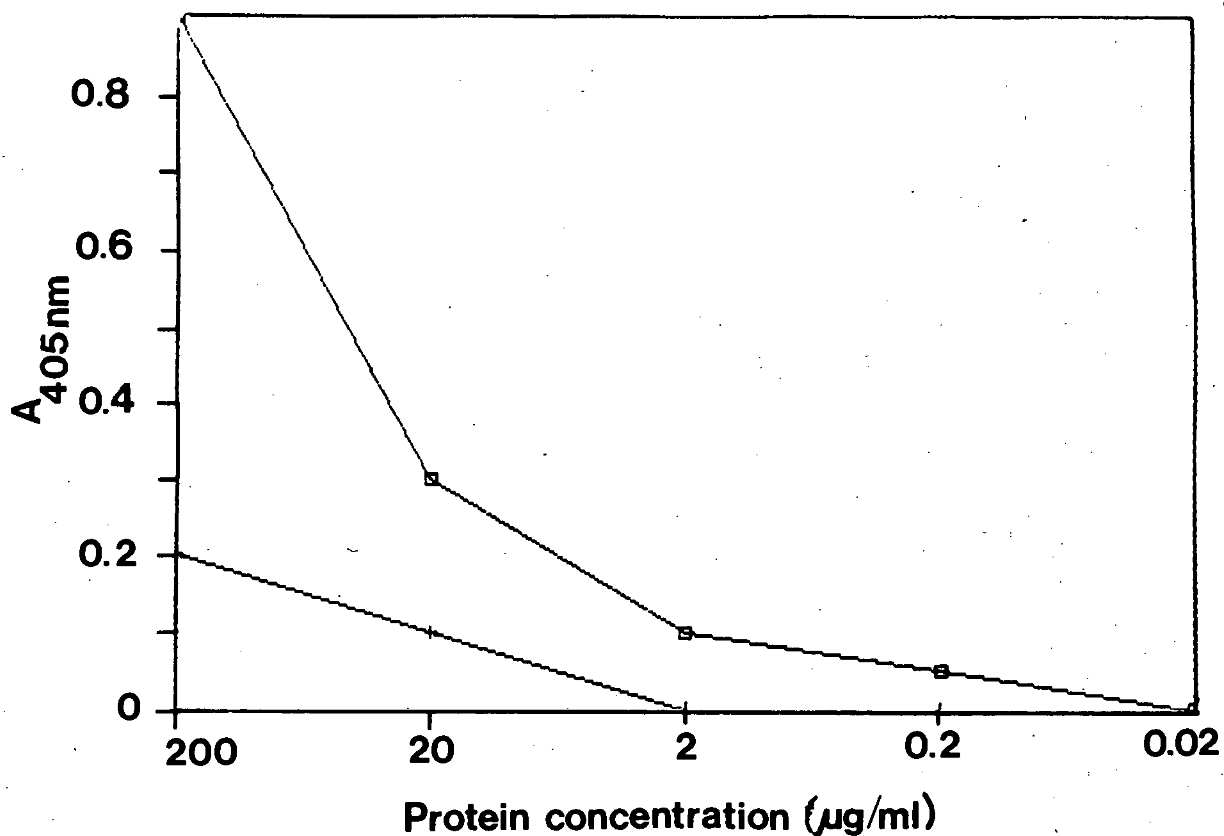


Fig. 3.5. ELISA absorbance values of _V. alginolyticus_ GS produced in _E. coli_ cells under conditions of high (□) and low (+) oxygen. Protein extracts were prepared from _E. coli_ YMC11(pRM210) grown at 30^{0}C in minimal medium supplemented with 15 mM glutamate and 0.15 mM glutamine. Aerated cultures (high oxygen) were incubated with shaking and unaerated cultures (low oxygen) were incubated without shaking.

3.3.4 Ntr phenotype. The Ntr genes (glnF, glnL, glnG) have been shown to control the level of arginine transport activity, the high-affinity glutamine transport system and the activation of the Klebsiella hut operon carried by E. coli ET8051 (Gracia et al., 1977; Kustu et al., 1979a; Kustu et al., 1979b; Pahel and Tyler, 1979; McFarland et al., 1981; Magasanik, 1982). The presence of complementary glnL and glnG genes on the cloned V. alginolyticus glnA DNA fragment in E. coli YMC11 was determined by growth on arginine or a low level of glutamine, and by monitoring the levels of histidase activity in cells grown under conditions of limiting and excess nitrogen. The E. coli YMC11 glnA, glnL, glnG deletion strain was unable to grow on minimal medium containing arginine or low levels of glutamine (Table 3.5). E. coli YMC10 wild-type and E. coli YMC11 containing pRM210 or pRM211 grew well on these media. As a result of the hutC mutation in the Klebsiella hut operon carried by E. coli YMC10 and YMC11 (Tuli et al., 1982), the basal level of histidase activity was significantly high in the absence of the normal inducer (Table 3.6). The hutC gene encodes a repressor whose natural inducer, urocanate, is produced by the first enzyme of the hut pathway (histidase) and is destroyed by the second enzyme of the pathway (urocanase) (Schlesinger et al., 1965; Prival and Magasanik, 1971). However, experiments utilizing E. coli YMC11 showed that this relatively high level of histidase activity was not affected by growth in limiting or excess nitrogen while E. coli YMC10 wild-type showed a 3-fold increase in histidase specific activity when grown in limiting nitrogen. The

Table 3.5. Plate tests for growth, of *E. coli* wild-type and *glnA*, *glnL*, *glnG* deletion strains with and without pRM210 or pRM211, on glutamine and arginine as nitrogen sources.

E. coli strains	Nitrogen source			Phenotype
	20 mM NH_4^+	0.5 mM glutamine	0.5 mM glutamine 10 mM arginine	
YMC11	-[a]	-	+	$GlnA^-$ Ntr^-
YMC11(pRM210)	+[b]	+	+	$GlnA^+$ Ntr^+
YMC11(pRM211)	+	+	+	$GlnA^+$ Ntr^+
YMC10	+	+	+	$GlnA^+$ Ntr^+

-[a], no growth ; +[b], growth. Growth was scored after 3 days incubation at 30^{0}C. All strains grew well on minimal medium supplemented with 15 mM glutamine as the sole nitrogen source.

Table 3.6. Effect of nitrogen levels on the expression of histidase activity in E. coli wild-type and glnA, glnL, glnG deletion strains with and without pRM210 or pRM211.

E. coli strains	Histidase activity		Phenotype
	0.15 mM glutamine	15 mM glutamine	
YMC11	0.1	0.1	Ntr^-
YMC11(pRM210)	0.1	0.1	Ntr^-
YMC11(pRM211)	0.1	0.1	Ntr^-
YMC10	0.6	0.2	Ntr^+

E. coli strains were grown in minimal medium containing 15 mM glutamate and 0.15 mM (limiting nitrogen) or 15 mM (excess nitrogen) glutamine at 30^{0}C with aeration. Histidase activity is expressed as μmol urocanate formed/min/mg protein.

introduction of pRM210 or pRM211 to E. coli YMC11 did not increase the level of histidase activity under conditions of limiting nitrogen.

Strains which have lost the ntrA function are glutamine auxotrophs (Gracia et al., 1977). The requirement for a functional glnF gene for V. alginolyticus glnA expression in E. coli was determined by the ability of the V. alginolyticus GS to complement a $ntrA^-$ mutation in E. coli. E. coli YMC22 $glnF^-$ mutant strains with and without pRM210 or pRM211 were unable to grow in minimal medium containing arginine as the sole nitrogen source (Fig 3.6). E. coli YMC10 wild-type grew well in this medium.

The possibility that the cloned V. alginolyticus glnA region coded for a gene product(s) which was capable of regulating expression of the E. coli GS in trans was examined. To achieve this, the E. coli glnA gene was sub-cloned into a low copy number ColE1 compatible plasmid designated pEGS (Fig. 3.2). The 2.8 kb fragment of E. coli specific DNA in pEGS contained a functional and constitutive glnA gene but no functional glnL or glnG genes. In addition, plasmids harbouring fragments of DNA spanning the glnA region of V. alginolyticus (Fig. 3.1) were constructed. Plasmid pRM213 was a deletion derivative of pRM210 in which an EcoRI endonuclease fragment internal to the V. alginolyticus glnA gene has been deleted (Chapter 2). Plasmid pVNTR1 contained a 3.7 kb HindIII restriction fragment of DNA from pRM210 and pVNTR2 contained a 2.75 kb SalI restriction fragment of DNA

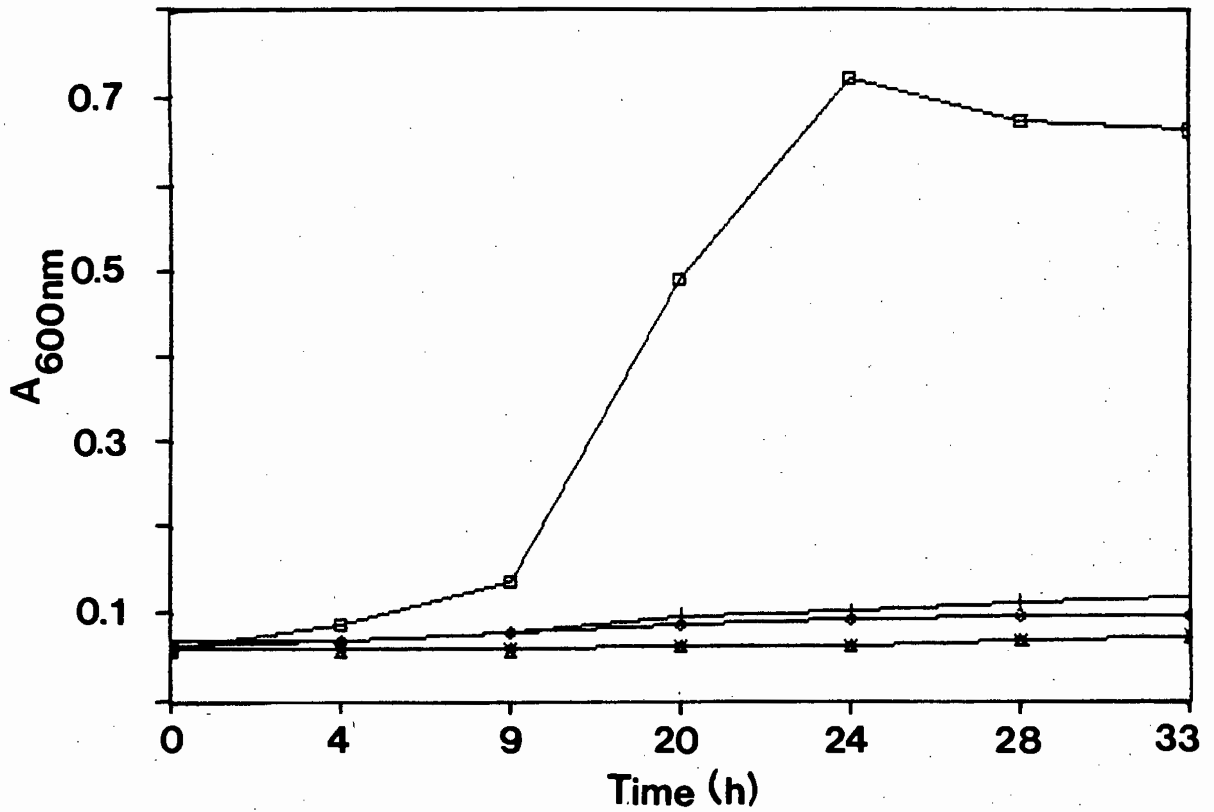


Fig. 3.6. Growth in minimal medium containing 10 mM arginine as sole nitrogen source of _E. coli_ YMC10 (□), _E. coli_ YMC22 _ntrA_⁻ mutant strain (◇) and _E. coli_ YMC22 containing pRM210 (+) or pRM211 (✱). Overnight cultures grown in Luria broth were washed twice, diluted into minimal medium and grown aerobically at 30^{0}C.

from pRM210. The 3.7 kb *Hind*III endonuclease and 2.75 kb *Sal*I endonuclease fragments of DNA were located downstream of the *V. alginolyticus glnA* gene. Plasmids pRM213, pVNTR1 and pVNTR2 were sub-cloned into *E. coli* YMC11(pEGS) and the effect of nitrogen levels on the expression of the *E. coli* GS activity was determined by comparing GS activity in cells grown at 30 and 37^{0}C respectively, under nitrogen-excess or nitrogen-limiting conditions (Table 3.7). *E. coli* YMC11 containing pEGS displayed low levels of GS specific activity under all experimental conditions (Table 3.7). The low constitutive expression of GS in *E. coli* YMC11(pEGS6) was subject to *in trans* regulation by plasmids pRM213 and pVNTR1. Introduction of pRM213 into *E. coli* YMC11(pEGS) resulted in an approximately 5-fold increase in the basal level of GS activity under conditions of excess-nitrogen (15 mM glutamine) at 30^{0}C (Table 3.7, line 2). This activity was further induced approximately 2.5-fold under conditions of limiting-nitrogen (15 mM glutamine). Similarly, expression of GS in *E. coli* YMC11(pEGS, pRM213) at 37^{0}C was induced approximately 5.5-fold, in comparison with the basal level of expression, under conditions of excess-nitrogen. This activity was induced approximately 2-fold under conditions of limiting-nitrogen. Expression of GS in *E. coli* YMC11(pEGS, pVNTR1) at 30^{0}C was not induced under conditions of excess-nitrogen (Table 3.7, line 3). This basal level of expression of GS was however, induced approximately 2.5-fold under conditions of limiting-nitrogen. Similar levels of GS activity were obtained for *E. coli* YMC11(pEGS, pVNTR1) grown at 37^{0}C. *E. coli*

Table 3.7. Effect of temperature and nitrogen levels on the expression of GS activity in E. coli YMC11 glnA⁻, glnL⁻, glnG⁻ containing pEGS6 and either pRM213, pVNTR1 or pVNTR2.

E. coli strains	GS activity			
	30^0C		37^0C	
	0.15 mM glutamine	15 mM glutamine	0.15 mM glutamine	15 mM glutamine
YMC11(pEGS)	0.35	0.36	0.33	0.29
YMC11(pEGS,pRM213)	4.02	1.70	3.29	1.59
YMC11(pEGS,pVNTR1)	0.67	0.27	0.69	0.26
YMC11(pEGS,pVNTR2)	0.31	0.25	0.22	0.28
YMC11(pRM213)	0	0	0	0
YMC11(pVNTR1)	0	0	0	0
YMC11(pVNTR2)	0	0	0	0

E. coli strains were grown overnight in minimal medium containing 15 mM glutamate and 0.15 mM glutamine as nitrogen sources. The cultures were diluted into fresh medium containing 15 mM glutamate and either 0.15 mM glutamine or 15 mM glutamine and grown aerobically at 30 or 37^0C for 2-3 generations before the cultures were harvested. GS specific activity is expressed as μmol γGT produced/min/mg protein.

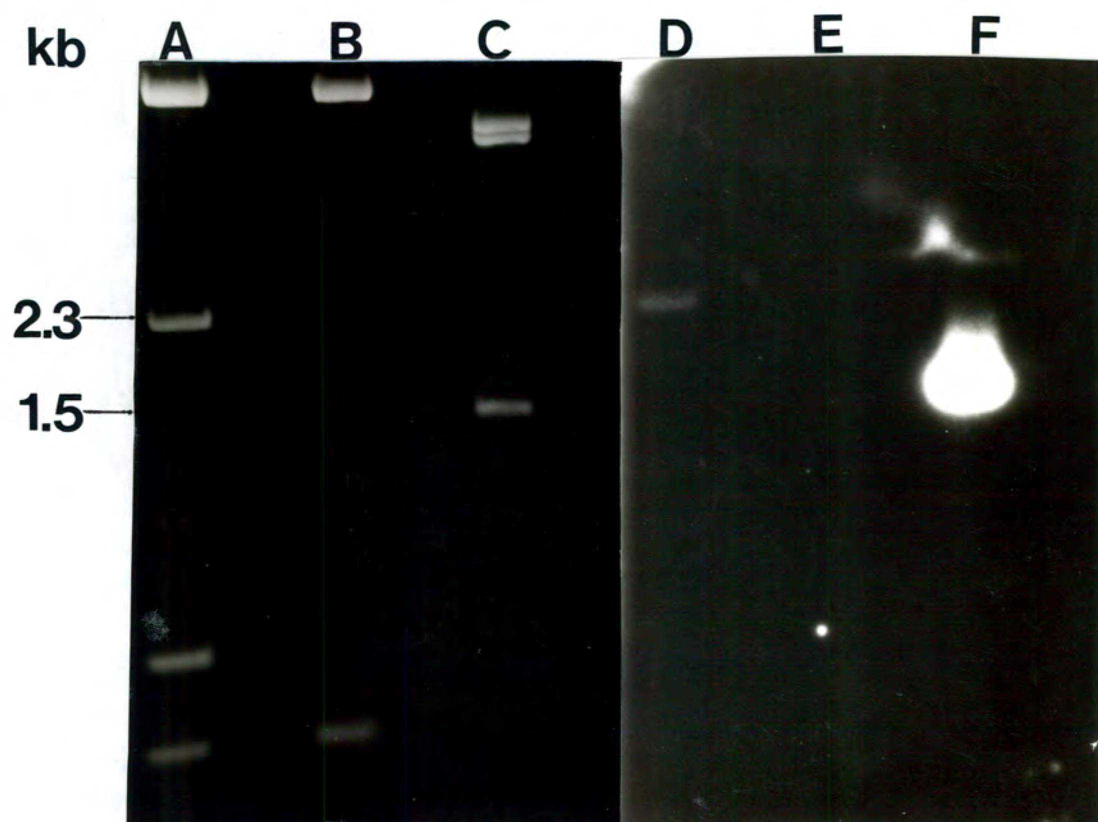


Fig. 3.7. Hybridization of λENTR with the glnA region of V. alginolyticus. Lanes A-C: Ethidium bromide stained agarose gel of DNA restriction endonuclease products. Lane A: SalI/HindIII digest of pRM210; laneB: SalI/HindIII digest of pRM211; laneC: PvuII digest of λENTR. Lanes D-F: autoradiogram of lanes A-C hybridized with [α-^{32}P]-labelled λENTR.

YMC11(pEGS, pVNTR2) produced low constitutive levels of GS under all experimental conditions (Table 3.7, line 4). E. coli YMC11 strains harbouring pRM213, pVNTR1 or pVNTR2 failed to express GS activity.

3.3.5 DNA homology. DNA homology between a sequence located downstream of the V. alginolyticus glnA gene and the glnL, glnG region of E. coli was demonstrated by Southern blotting and DNA hybridization (Fig. 3.7). The phage λENTR harbouring a 1.5kb PvuII restriction fragment of DNA from p804 (Fig. 3.2), spanning the carboxy-terminus of the glnL gene and the amino-terminus of the glnG gene of E. coli, was used as a probe. The [α-^{32}P]-labelled probe hybridized with a single 2.3kb SalI/HindIII restriction fragment of V. alginolyticus genomic DNA present in pRM210 but not in pRM211 (Fig. 3.7, lanes D and E respectively). A single 1.5kb fragment from the PvuII digest of p804 hybridized with the probe (Fig. 3.7, lane F).

3.4 DISCUSSION

An interesting aspect of the production of the collagenase and serine proteases by V. alginolyticus is that their synthesis is specifically regulated by temperature and oxygen as well as nitrogen availability (Hare et al., 1981). The expression of the cloned V. alginolyticus GS in an E. coli glnA, glnL, glnG deletion strain was also subject to regulation by oxygen temperature and nitrogen availability. There was no difference in the stability of GS at 30 or 37^{0}C

since preformed enzyme maintained at 30 and 37^{0}C respectively was inactivated at the same rate. The different levels of GS activity detected under the various growth conditions were indeed due to differential expression of the *glnA* gene. Enzyme-linked immunosorbent assays for the detection of *V. alginolyticus* GS showed that the amount of antigen produced was proportional to the GS specific activity estimated by the γGT assay. Reduced levels of antigen were produced at 37^{0}C and under conditions of low oxygen.

The conclusion that the observed oxygen and temperature regulated expression of the cloned *V. alginolyticus* GS in *E. coli* was mediated via a *V. alginolyticus* DNA regulatory region adjacent to the *V. alginolyticus* *glnA* gene in pRM210 was based on the following observations; (i) the GS specific activity in an *E. coli* wild-type strain was not affected by temperature or oxygen; (ii) The presence of pcI857 containing a temperature sensitive lambda repressor gene did not affect the regulation of the *V. alginolyticus* gene by temperature or oxygen. Therefore, the temperature and oxygen regulated expression of the *V. alginolyticus* GS was not regulated by the temperature sensitive lambda rightward promoter. Regulation of the *V. alginolyticus* GS by the lambda rightward promoter should have resulted in a repression of GS activity at 30^{0}C in contrast to the observed increase in GS expression at 30^{0}C; and (iii) deletion of a 3.27 kb *V. alginolyticus* DNA fragment from the area adjacent to the lambda rightward promoter on pRM210

yielded pRM211 which produced very low levels of GS activity under all the experimental conditions and expression of GS was not affected by temperature or oxygen. The expression of GS activity in E. coli wild-type and E. coli YMC11(pRM210) were subject to regulation by nitrogen availability. However, the low levels of GS activity expressed from pRM211 were not subject to nitrogen regulation. The regulatory region therefore controlling the expression of the V. alginolyticus glnA gene by temperature, oxygen or nitrogen was assumed to be situated on the 3.27 kb fragment which was deleted in pRM211. The low level of GS activity produced by pRM211 was presumably due to either basal levels controlled from some remaining regulatory sequences on pRM211 or read through from a regulatory region on the pEcoR251 fragment of pRM211. As pcI857 did not affect the low levels of the GS expression by pRM211 it would appear that the lambda rightward promoter is not involved in the low level of GS expression in pRM211. M_r determinations from the Western blotting experiments indicated that pRM211 codes for an intact gene product and a truncated polypeptide is not produced (Chapter 2, Fig. 2.3).

In E. coli and S. typhimurium the glnA, glnL and glnG genes are adjacent to each other on 1.5, 1.0 and 1.7 kb fragments respectively. Tuli et al. (1982) were able to determine the phenotype of strains with respect to Ntr genes by scoring for growth on arginine or a low level of glutamine and by plasmid complementation for hut gene activation. Results obtained in this study however, indicate that the the

criteria used by Tuli et al. (1982) for the determination of Ntr phenotype does not always hold true. E. coli strains deleted for glnA, glnL and glnG but carrying pRM210 or pRM211 were able to grow on minimal medium containing low levels of arginine or glutamine as sole nitrogen source. This would suggest that both pRM210 and pRM211 contained functional (analogous) glnL and glnG genes on the cloned V. alginolyticus DNA fragment. The deletion derivative pRM211 contains a 2.4 kb fragment of V. alginolyticus DNA. A fragment of this size would not be large enough to accommodate the glnA, glnL and glnG genes. E. coli YMC11 glnA, glnL, glnG deletion strain containing pRM211 produced low constitutive levels of GS under all experimental conditions. The low levels of GS produced by pRM211 are sufficient for growth on minimal medium containing low levels of arginine or glutamine as sole nitrogen source. It would appear that the system employed by Tuli et al.(1982) is useful only under circumstances where the regulation of GS expression is 100%. Strains that displayed a Ntr$^+$ phenotype on the plate test would be expected to activate hut gene expression (Tuli et al., 1982). The introduction of pRM210 or pRM211 into E. coli YMC11 did not increase the level of histidase activity under conditions of limiting nitrogen. These results were contradictory and did not establish decisively whether the 5.7 kb DNA fragment of the glnA region of V. alginolyticus contained genes which were analogous to the glnL and glnG genes present in other Gram-negative bacteria. Gracia et al (1977) have shown that E. coli strains that have lost the glnF function are glutamine

auxotrophs. *E. coli* YMC22 is an *glnF*$^-$ mutant strain unable to grow in minimal medium containing arginine as the sole nitrogen source. *E. coli* YMC22 containing pRM210 or pRM211 was unable to grow in minimal medium containing arginine as the sole nitrogen source. This finding implied the possible involvement of a functional *glnF* gene in the expression of the cloned *V. alginolyticus* GS.

Southern blotting and DNA hybridization was used to demonstrate homology between a DNA sequence located downstream of the *glnA* gene in pRM210 and the *glnL,glnG* region of *E. coli*. The products of the *glnL* and *glnG* genes of the enteric bacteria are involved in the cascade regulation of GS (Chapter 1.2). *In trans* regulatory studies were carried out in order to establish whether the DNA sequences located downstream of the *V. alginolyticus glnA* gene in pRM210 encoded genes which were functionally analogous to the *glnL* and *glnG* genes of *E. coli*. Plasmids pRM213 and pVNTR1 were able to complement the *glnL,glnG* deletion in *E. coli* YMC11(pEGS) at 30 and 30^{0}C. Although both pRM213 and pVNTR1 were capable of regulating the *E. coli* GS *in trans* with respect to nitrogen availibity, higher levels of GS activity were produced in *E. coli* YMC11(pEGS, pRM213) than in *E. coli* YMC11(pEGS, pVNTR1) at 30 and 37^{0}C. Plasmid pRM213 is a deletion derivative of pRM210 in which an *Eco*R1 restriction fragment internal to the *glnA* structural gene has been deleted. pRM213 has a GlnA$^-$ phenotype but retains *V. alginolyticus* DNA sequences located upstream of the *glnA* gene. Plasmid pVNTR1 contains only those sequences which are located downstream of the *V.*

alginolyticus glnA gene. The enhanced levels of GS activity in E. coli YMC11(pEGS, pRM213) could be due to higher levels of putative glnL and glnG genes resulting from read-through from the glnA promoter in pRM213 (Reitzer and Magasanik, 1985). The ability of plasmids pRM213 and pVNTR1 to regulate expression of the E. coli GS in trans was independent of the growth temperature, suggesting that expression of the regulatory proteins was not repressed at 37^0C as is the case for the V. alginolyticus GS.

In K. pneumoniae, the expression of Nif genes is regulated at the transcriptional level by the glnF, glnG,and nifLA gene products (Buchanan-Wollaston, 1981; Hill et al., 1981; Merrick et al., 1982; Sundaresan et al., 1983). The product of nifA in conjunction with that of glnF activates all other nif operons in the absence of high temperature (Ow and Ausubel, 1983; Brooks et al., 1984). The nifL product acts as a repressor for Nif genes in the presence of oxygen or an excess of fixed nitrogen (Hill et al., 1981; MacNeil et al., 1981; Filser et al., 1981). In a recent study, Kong et al. (1986) have suggested that oxygen repression of nifL is probably not mediated by the product of the glnG gene. Using lacZ fusions these workers were able to show that expression of lacZ from the nifL promoter in an E. coli YMC11 glnA, glnL, glnG deletion strain was repressed by oxygen. The oxygen effect on nifL-lacZ expression is apparently due to the high sensitivity of the nifL promoter to oxygen. The molecular mechanism of this sensitivity to oxygen is as yet unknown.

Expression of the cloned V. alginolyticus glnA gene was subject to regulation by oxygen, temperature and nitrogen levels in an E. coli glnA, glnL, glnG deletion strain. Proteins expressed from a region downstream of the glnA gene of V. alginolyticus were able to regulate expression of the E. coli GS in trans with respect to nitrogen availability. Expression of the regulatory proteins was apparently unaffected by the growth temperature. It is therefore tempting to speculate that the oxygen and temperature regulated expression of V. alginolyticus GS in E. coli is due to sensitivity of the V. alginolyticus glnA promoter to oxygen and elevated temperatures. Attempts to obtain promoter fusions with Mini-Mu bacteriophage transposons (Castilho et al., 1984) were, however, unsuccessful. The precise mechanism of oxygen and temperature regulated expression of the V. alginolyticus GS, collagenase and exoprotease genes has yet to be determined. Bodasing et al. (1985) have previously shown that the V. alginolyticus GS is regulated by an adenylylation-deadenylylation system. The current study provides evidence for the occurrence of genes which are functionally analogous to the Ntr genes of E. coli.

CHAPTER FOUR

NUCLEOTIDE SEQUENCE OF *V. alginolyticus* *glnA* and *glnL* genes

CHAPTER FOUR

NUCLEOTIDE SEQUENCE OF *V. alginolyticus* *glnA* AND *glnL* GENES

Summary. The nucleotide sequence of a 3.5 kb fragment containing the *V. alginolyticus* *glnA* and *glnL* genes was determined. The *glnA* and *glnL* genes occupied regions of 1.4 and 1.0 kb respectively, with a 0.75 kb intergenic region. The upstream region of the *glnA* gene contained tandem promoters. The upstream promoter resembled the consensus sequence for *E. coli* σ^{60} promoters whereas the presumptive downstream promoter showed homology with Ntr regulated promoters. Expression of *glnA* from the upstream promoter produced a transcript with an untranslated leader sequence of 267 nucleotides. Four putative NR_I binding sites were located between the tandem promoters. The *glnL* gene was preceded by a single putative NR_I binding site. A third ORF was located 45 bp downstream from the *glnL* gene. The amino acid sequence deduced from this sequence was homologous to the N terminal end of the *E. coli* NR_I protein. The deduced amino acid sequences of the *V. alginolyticus* *glnA* and *glnL* genes were analyzed and compared with the reported amino acid sequences of GS and NR_{II} proteins from other organisms. It is concluded that the *V. alginolyticus* *glnA* gene is located on an operon with components that are structurally similar to the *glnALG* operon of the enteric bacteria.

4.1 INTRODUCTION

Genes involved in the Ntr system of enteric bacteria have been discussed in Chapter 1. Recent evidence suggests that the Ntr system may be conserved among diverse bacterial species. Toukdarian and Kennedy (1986) have characterized genes which are functionally analogous to the glnF and glnG genes of E. coli from the non-enteric bacterium Azotobacter vinelandii. The glnG gene was found to be adjacent to the glnA gene but glnF, as in the enteric bacteria, was not linked to glnA. Products of both the glnF and glnG genes were required for nitrate utilization. In contrast with K. pneumoniae, where both glnF and glnG are essential for expression of Nif genes, in A. vinelandii glnF but not glnG is required.

The cloning of glnG genes and the creation of glnG mutations have also been described for the plant associated bacterial species belonging to the Rhizobiaceae (Nixon et al., 1986; Pawlowski et al., 1987; Rossbach et al., 1987; Szeto et al., 1987; Carlson et al., 1987). The Rhizobiaceae family includes members of the genera Rhizobium, Bradyrhizobium, Agrobacterium and Phyllobacterium. Two GS enzymes designated GSI and GSII are produced by members of this family. GSI is similar to the single GS enzyme found in most other Gram-negative bacteria. It is a polymeric enzyme consisting of twelve identical subunits, is relatively heat stable and is regulated by reversible adenylylation and by feedback inhibition (Darrow and Knotts, 1977; Darrow, 1980).

GSII is similar to the eukaryotic enzyme and lacks the C terminal domain of the prokaryotic enzyme (Darrow and Knotts, 1977; Filser _et al_., 1986). The enzyme has eight subunits, is heat labile and is not subject to post-translational modification (Darrow and Knotts, 1977; Darrow, 1980). The two proteins are products of different genes and are differentially regulated in response to nitrogen availability, carbon source and oxygen concentration (Rao _et al_., 1978; Darrow, 1980; Ludwig, 1980; Darrow _et al_., 1981; Somerville and Kahn, 1983). Edmands _et al_. (1987) have isolated two GS enzymes from the actinomycete _Frankia_ Cp11 which show similar properties and regulation to GSI and GSII of the _Rhizobiaceae_. This finding lends support to the proposal that symbiotic bacteria have evolved unique modes of nitrogen metabolism in addition to the systems found in freeliving bacterial species (Edmands _et al_., 1987).

Carlson _et al_., (1987) have shown that a dual-promoter control of GS expression is found in _B. japonicum_, with the interesting difference that each promoter directs the expression of a separate GS gene. GSI is encoded by the _glnA_ gene and is transcribed from a single promoter that is similar to the bacterial consensus promoter. The level of transcription of _glnA_ is not affected by nitrogen availability in the growth medium. GSII is transcribed from a single promoter in the _glnII_ gene. The _glnII_ promoter has structural features characteristic of promoters controlled by the Ntr system, initiation of transcription from the _glnII_ promoter is regulated in response to nitrogen

availability. The *glnG* gene is essential for transcriptional activation of the *A. tumefaciens* C58 and the *R. meliloti* 1021 *glnII* gene but not the *glnII* gene (Rossbach *et al*., 1987). This suggests that the dual-promoter control of GS expression could occur more widely in the *Rhizobiaceae* family. Another peculiarity of members of this family is that the *glnB* gene, the structural gene for P_{II}, is apparently contiguous to the gene encoding GSI (Colonna-Romano *et al*., 1987). Evidence to date indicates that the *glnB* gene of *E. coli* is unlinked to *glnA* and forms part of an as yet unidentified operon (Son and Rhee, 1987). The deduced amino acid sequence of the *R. leguminosarum* P_{II} protein shares 50% homology with the *E. coli* protein when optimally aligned. The *glnB* gene of *R. leguminosarum* is preceded by the consensus sequence for a nitrogen-regulated promoter (Colonna-Romano *et al*., 1987), but the transcriptional start(s) of the *glnB* and *glnA* genes have however not as yet been confirmed.

Results obtained in Chapter 3 suggested that the *V. alginolyticus glnA* region encoded polypeptides that were functionally analogous to the *E. coli glnL* and *glnG* genes. A 3.5 kb fragment of the *V. alginolyticus glnA* region from pRM210 was sequenced to determine the structural organization of this region and the extent of homology to the *E. coli glnL* and *glnG* genes.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains, plasmids and phages. The bacterial strains are listed in Table 4.1. Plasmids and phages are listed in Table 4.2. E. coli strains were grown in Luria medium (Miller, 1972) and glucose minimal medium (Miller, 1972) supplemented with either $(NH_4)_2SO_4$ (20 mM) or glutamate (15 mM) and glutamine (0.15 mM) as the nitrogen source.

4.2.2 Construction of plasmids and phages. Standard techniques for manipulation of DNA (Maniatis et al., 1982) were used (Appendix C). Restriction maps of vectors are given in Appendix F. The M13 phage vectors and the plasmid pUC18 (Messing and Vieira, 1982) were obtained from Bethesda Research Laboratories (BRL Inc., USA) and the phagemid (bluescript, KS) was obtained from Stratagene Cloning Systems, San Diego, USA. Plasmid pRM210 has been described in Chapter 2. The construction of pVNTR1 has been described in Chapter 3. Plasmids pRM214, pRM215, and M13 clones Eco1 (Fig. 4.1) and GSP (Fig. 4.2) were constructed in E. coli JM105 by insertion into multiple cloning sites within the lacZ genes of the vectors. The phage Eco1 resulted from the insertion of a 0.9 kb EcoRI restriction fragment of V. alginolyticus DNA from pRM210 into the EcoRI restriction site of M13-mp18. Bacteriophage GSP is a sub-clone of pVGS500 harbouring the promoter region of the V. alginolyticus GS structural gene. Construction of GSP involved the ligation of a 1.1 kb PstI/EcoRI endonuclease

Table 4.1. Bacterial strains

Strain	Relevant genotype	Reference or source
Escherichia coli		
YMC11	$glnA^-$,$glnL^-$,$glnG^-$	Backman *et al*.(1981)
JM105	*thi*,*rpsL*,*endA*, *sbcB15*,*hspR4*, (lac-pro AB), [F$'$,*tra*36,proAB *lacI*^{q} Z M15]	Yanish-Perron *et al*.(1985)

Table 4.2. Plasmids and phages

Plasmid	Description	Reference or source
pUC18	Ap^r	Messing and Vieira(1982)
Bluescript (KS)	Ap^r	Stratagene Cloning Systems, San Diego, USA
pRM210	*glnA*$^+$,Ap^r	Current study
pRM214	*glnA*$^+$,Ap^r	Current study
pRM215	*glnA*$^-$,Ap^r	Current study
pVNTR1	*glnA*$^-$,Ap^r	Current study
pVGS500	*glnA*$^+$,Ap^r	E Rumbak(1988)

Bacteriophage	Description	Reference
M13-mp18	Ap^r	Messing and Vieira(1982)
M13-mp19	Ap^r	Messing and Vieira(1982)
*Eco*1	*glnA*$^-$,Ap^r	Current study
GSP	*glnA*$^-$,Ap^r	Current study

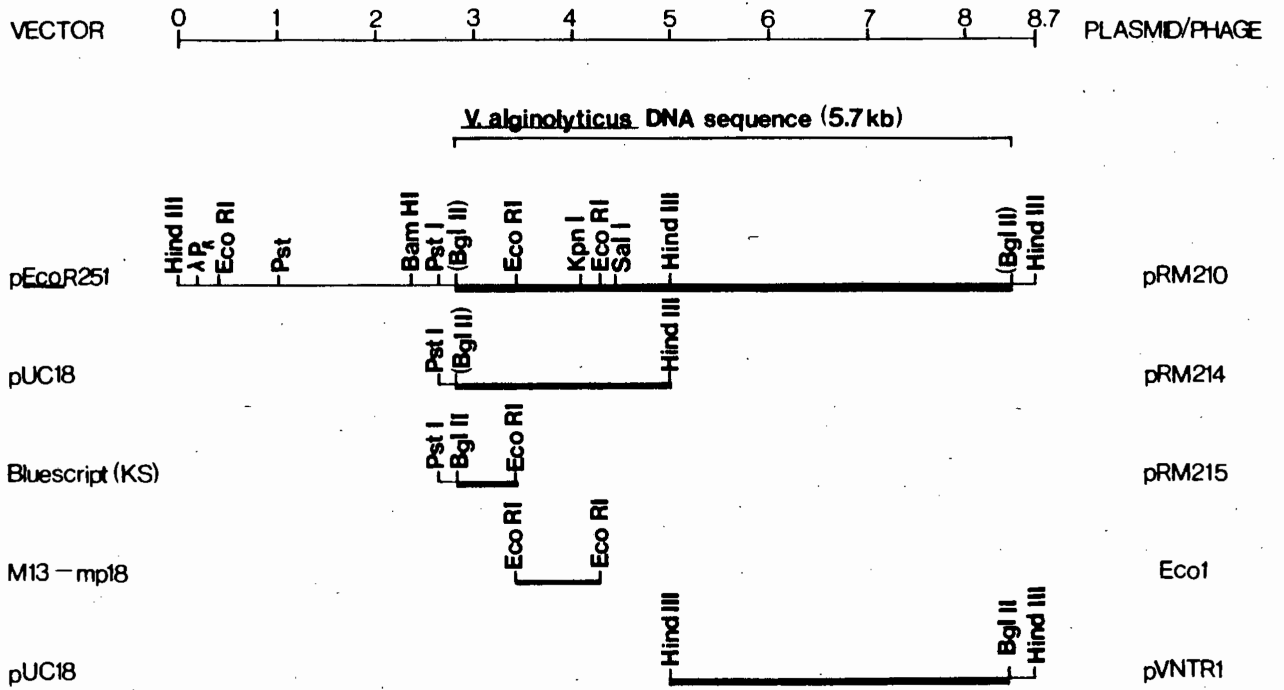


Fig. 4.1. Restriction map and sub-clones of pRM210

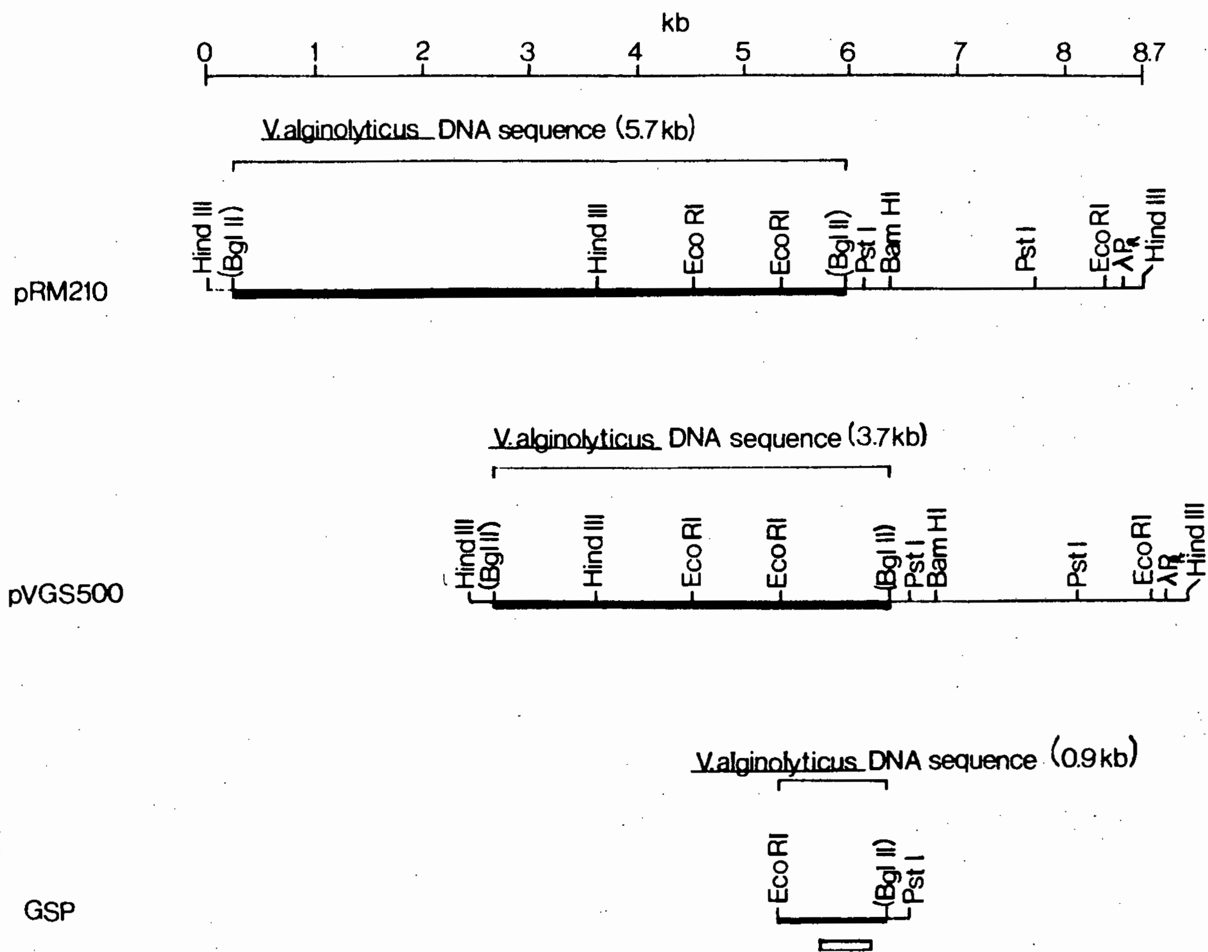


Fig. 4.2. Restriction maps of pRM210, pVGS500 and GSP. The open bar indicates the V. alginolyticus glnA promoter region.

fragment from pVGS500 with PstI/EcoRI endonuclease linearized M13-mp19. Ligation mixes were transformed into competent E. coli JM105 cells (Appendix C) and lac^- Ap^r transformants were selected on Luria agar (plasmids) or H agar (phages) supplemented with 100 μg/ml Ap, 0.2 mM IPTG and 0.004% (w/v) Xgal. All plasmid constructions were verified by restriction endonuclease analysis and agarose gel electrophoresis.

4.2.3 Sequencing strategy. Plasmid pRM210 which contained the V. alginolyticus glnALG operon was used as the initial source of DNA for sequencing. The sequencing strategy employed is outlined in Fig. 4.3. Fragments suitable for sequencing were generated by constructing subclones of pRM210 in bacteriophage M13 strains mp18 and mp19 and plasmids pUC18 and bluescript (KS). Ordered sets of overlapping deletions were obtained by exonuclease III digestion of selected subclones (Henikoff, 1987).

4.2.4 Targeting of deletions. A modification of the method of Henikoff (1987) was employed to generate ordered sets of deletion breakpoints for DNA sequencing (Appendix C). Four subclones (pRM214, pRM215, Eco1 and pVNTR1) which were shortened by exonuclease III activity are diagrammatically represented in Fig. 4.1 and Fig. 4.4. Fragments of DNA from pRM210 were inserted into the vector such that cleavage by two enzymes in the polylinker region of the vector would lead to a 5'-protrusion adjacent to the insert and a 3'-protrusion adjacent to the sequencing primer site (Table

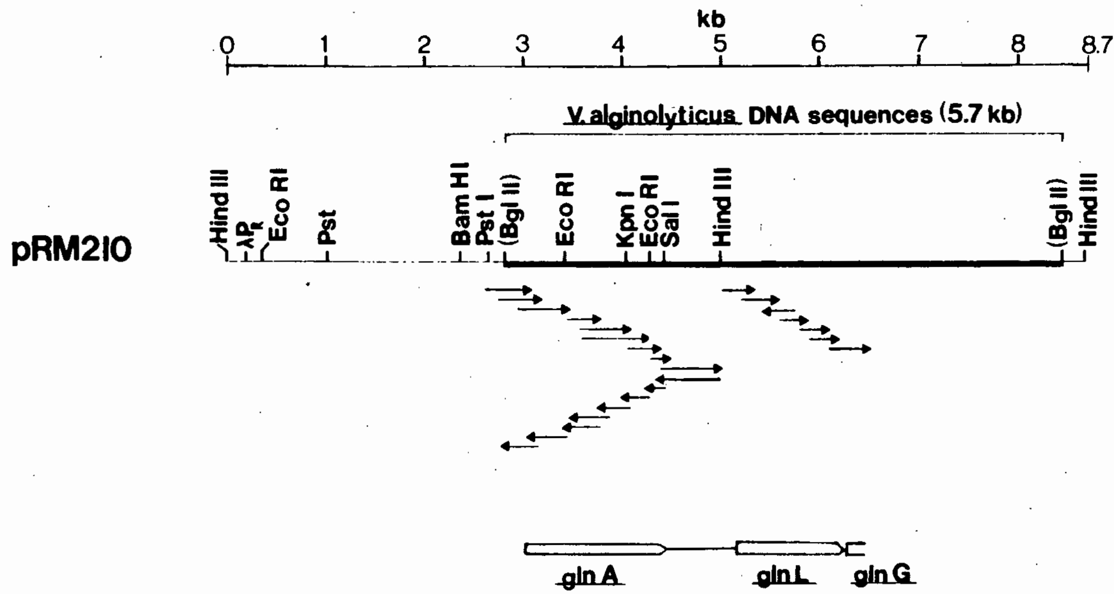


Fig. 4.3. Structural organization of the V. alginolyticus glnA region. Arrows indicate the extent and direction of DNA sequence determination.

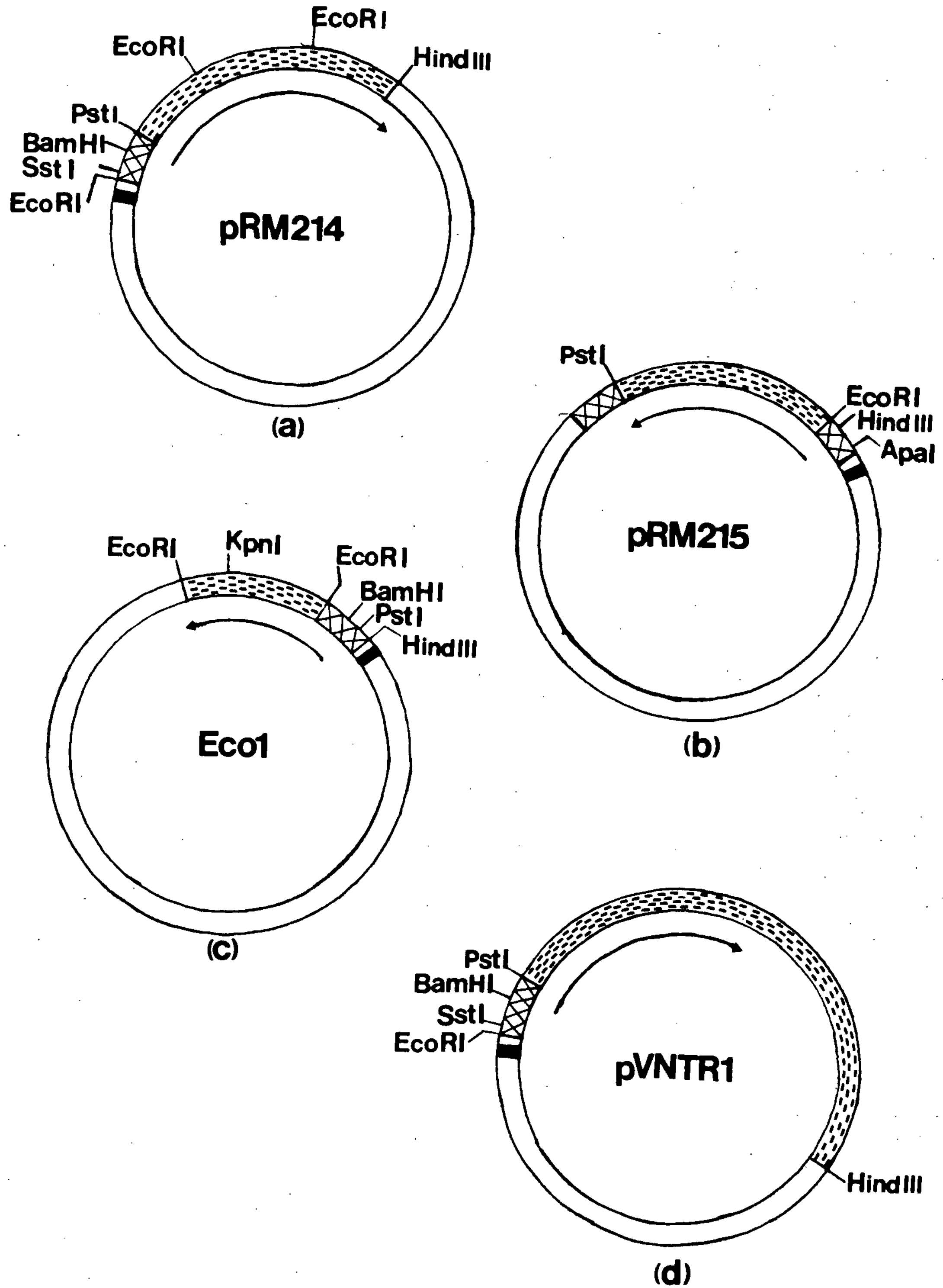


Fig. 4.4. Diagramatic representation of sub-clones constructed for targeting of ordered deletions employing exonuclease III. The relevant restriction endonuclease sites, insert DNA (-.-.-.), the polylinker region of the vector (XXX) and the sequencing primer site are shown.

4.3). The four-base 3'-protrusion protected the vector from exonuclease III attack allowing unidirectional digestion of the insert sequence from the 5'-protrusion left by the other enzyme. Samples were removed at timed intervals, treated with S1 nuclease, Klenow DNA polymerase, T4 DNA ligase and used to transform competent E. coli cells (Appendix C). Deletion endpoints were determined by restriction analysis (Table 4.3) and agarose gel electrophoresis.

4.2.5 Nucleotide sequence determination. Preparation of double (plasmid) and single stranded (phage) template DNA for nucleotide sequence determination is described in Appendix C. DNA sequences were determined by the dideoxynucleoside triphosphate chain termination method of Sanger et al. (1977). Reagents for sequencing reactions were obtained as a kit and used according to the manufacturers specifications. The BRL (BRL Inc., USA) and Sequenase (US Biochemical Corporation) kits were used. Two M13-specific sequencing primers were used. The 14-mer reverse primer (5'-TCCAGTCACGACGT-3') and the 17-mer forward primer (5'-TCCCATTTCCGCCAG-3') were purchased from Amersham Int., UK. In addition, one custom-made primer was synthesized ("D Botes", Dept of Biochemistry, University of Cape Town, SA) in a Beckman System 1 plus DNA synthesizer (version 2.1). The custom-made 15-mer (5'-CAAATTCTGGAAAAG-3') was used to sequence a region in the glnL structural gene. The DNA chains were radiolabelled with [α-^{32}P]dCTP (3000 Ci/mmol) or [α-^{35}S]dCTP (400 Ci/mmol) obtained from Amersham Int., UK. Dideoxy sequencing reaction products

Table 4.3. Restriction enzymes used to generate ordered deletions in pRM214, pRM215, EcoI, and pVNTR1.

Plasmid or Phage	Restriction endonuclease		
	Enzyme A[a]	Enzyme B[b]	Enzyme C[c]
pRM214	BamHI	SstI	EcoRI
pRM215	HindIII	ApaI	PstI
Eco1	BamHI	PstI	EcoRI
pVNTR1	BamHI	SstI	HindIII

[a] Restriction enzyme used to generate a 5'-protrusion adjacent to the insert DNA.

[b] Restriction enzyme used to generate a 3'-protrusion adjacent to the sequencing primer site.

[c] Restriction enzyme used to determine deletion breakpoints in subclones

were analyzed in denaturing polyacrylamide gels (6% (w/v)). Polyacrylamide wedge gels 0.2 mm in thickness were cast in a BRL type apparatus. Resolved nucleotides were visualized by autoradiography. Gels containing [^{32}P]DNA were exposed under Cornex 4 autoradiographic film in an X-ray cassette containing a Fuji X-ray intensifying screen and exposed for 5-24 h. Gels containing [^{35}S]DNA were autoradiographed under Kodak XAR-5 X-ray film for 15-120 h.

4.2.6 DNA sequence analysis. The DNA sequences were analyzed with the IBM XT computer, DNA tools and Genepro (version 3.1) programs. Deduced amino acid sequences were analyzed and compared with the IBM XT Microgenie (version 299) protein alignment subroutine.

4.2.7 Isolation of RNA. A modification of the method of Aiba _et al_. (1981) was employed for extraction of total cellular RNA from _E. coli_ cells. The precautions taken to ensure that all glassware, plasticware and solutions used were RNase-free were as recommended by Maniatis _et al_. (1982) (Appendix A). _E. coli_ YMC11 containing GSP was grown overnight in minimal medium containing 15 mM glutamate and 0.15 mM glutamine as nitrogen sources. The overnight culture was diluted into 100 ml of fresh medium and grown aerobically at 30^{0}C for 2-3 generations. The following protocol describes the isolation of RNA from 20 ml of a mid-exponential phase culture. Cells were harvested (5000 rpm for 5 min) and immediately resuspended in 300 μl of lysis buffer (0.02 M sodium acetate, 0.5% (w/v) SDS, 1 mM EDTA, pH

5.5). An equal volume of water-saturated phenol was added and the mixture was incubated at 60^0C for 5 min with gentle shaking. After centrifugation (5000 rpm for 5 min) the aqueous phase was re-extracted with phenol (300 μl). The RNA was precipitated by adding 3 vol ethanol to the aqueous phase and chilled at -20^0C for 2h. The RNA pellet was recovered by centrifugation in an Eppendorf microfuge and resuspended in 300 μl of Lysis buffer. The ethanol precipitation was repeated twice and the final precipitate was resuspended in 100 μl of distilled water. Contaminating DNA was removed by treatment with RNase-free DNase (Boehringer Mannheim, West Germany) in accordance with the manufacturers specifications. The RNA was phenol extracted, ethanol precipitated and resuspended in 50 μl distilled water. RNA concentration was estimated spectrophotometrically. One absorbance unit (260 nm) was considered equivalent to 36 μg RNA/ml.

4.2.8 Detection of mRNA by S1 nuclease protection. A strand-specific probe for S1 nuclease mapping was prepared using single-stranded phage DNA from GSP as a template and a custom-made primer (VGS5) for primed synthesis by Sequenase. The filamentous phage GSP was a subclone of pVGS500 containing the non-coding strand of the <u>V. alginolyticus</u> GS structural gene. The 21-mer primer VGS5 (5'-AAGTTCTATCGCTCATCCAAC-3') was synthesized by "D Botes" (Dept of Biochemistry, University of Cape Town, SA) in a Beckman System 1 plus DNA synthesizer (version 2.1). Primer VGS5 would correspond to positions +14 to +34, of the GS

structural gene, according to the coordinate system used in Fig. 4.10. The strand-specific DNA probe was prepared in the presence of [α-^{32}P]dCTP essentially as described for sequenase reactions except that unlabeled dCTP was omitted. Reactions were performed at room temperature in a final volume of 15 μl. After 5 min incubation, unlabeled dNTP's (30 μM) were added to generate a high molecular weight probe. The chase reaction was continued for 5 min and was terminated with the addition of 1 μl of 0.1 M EDTA (pH 8.0). The reaction products were analyzed on 6% (w/v) denaturing polyacrylamide gels.

For S1 exonuclease analysis, 0.7 μl of probe and 200 μg of RNA were used. Probe-RNA hybridization was carried out in 30 μl hybridization buffer (20 mM HEPES pH 6.5, 0.4 M NaCl, 80% (v/v) formamide). Prior to hybridization, the mixture was heated to 80^{0}C for 10 min. Hybridization was carried out at 50^{0}C for 6 h. The mixture was diluted 10-fold with cold S1 buffer (30 mM sodium acetate pH 4.5, 3 mM zinc chloride, 300 mM NaCl) and nucleic acids were digested with 0.1-4.0 units/μl of S1 nuclease (Boehringer Mannheim, West Germany) at 37^{0}C for 30 min in the presence of carrier DNA (10 ng/μl, denatured salmon sperm DNA) (Appendix). The S1 nuclease digest was phenol extracted, ethanol precipitated, and resuspended in 5 μl of water and 5 μl of formamide dye solution (95 % (v/v) formamide, 20 mM EDTA, 0.05 % (w/v) bromophenol blue, 0.05 % (w/v) xylene cyanol). The digestion products were analyzed on 6 % (w/v) polyacrylamide

gels. Size markers were [α-^{32}P] labelled dideoxy sequencing products of GSP DNA primed with VGS5 primer.

4.3 RESULTS AND DISCUSSION

4.3.1 Nucleotide sequence of the *V. alginolyticus glnA* structural gene. The complete nucleotide sequence of the *glnA* structural gene is given in Fig. 4.5. The ORF presented in Fig. 4.5 codes for a protein of 468 residues with a calculated M_r of 52,3 kD. The deduced GS amino acid sequence of *E. coli* and *V. alginolyticus* were compared (Fig. 4.5). The *E. coli glnA* gene codes for a protein of 469 amino acid residues with a calculated M_r of 52,4 kD. The calculated M_r of the *E. coli* GS is in good agreement with the value of 50 kD estimated from Western blotting experiments (Chapter 2). The calculated M_r of the *V. alginolyticus* GS polypeptide encoded by pRM210 is, however, considerably lower than the predicted value of 62 kD determined by Western blotting. The apparent M_r of the purified GS polypeptide isolated from wild-type *V. alginolyticus* did not differ from that encoded by plasmid pRM210.

4.3.2 GS amino acid sequence homology. *V. alginolyticus* GS shares considerable homology with published data for the *E. coli* polypeptide. The two sequences share approximately 80% homology (Fig. 4.5). The sequence homology, which extends the entire length of the polypeptide (Fig. 4.6), increases to 89% when conserved amino acid residues are included.

```
   1  ATG TCA GTA GAA AAA GTT CTA TCG CTG ATC CAA GAA AAC GAA GTT AAG TTT GTT GAC CTA CGC TTC ACT GAT ACA AAA GGT AAA GAG CAG
   1   M   S   V   E   K   V   L   S   L   I   Q   E   N   E   V   K   F   V   D   L   R   F   T   D   T   K   G   K   E   Q

  91  CAC ATT TCG ATC CCT GCT CAC CAA ATC GAC GCA GAC TTC TTC GAA GAA GGT AAA ATG TTC GAT GGT TCA TCA GTT GCT GGC TGG AAA GGT
  31   H   I   S   I   P   A   H   Q   I   D   A   D   F   F   E   E   G   K   M   F   D   G   S   S   V   A   G   W   K   G

 181  ATC AAC GAA TCA GAC ATG GTA ATG ATG CCT GAC GCA TCT TCT GCT GTG CTT GAC CCA TTC ACG GAA GAC GCA ACA CTA AAC ATC CGT TGT
  61   I   N   E   S   D   M   V   M   M   P   D   A   S   S   A   V   L   D   P   F   T   E   D   A   T   L   N   I   R   C

 271  GAC ATC TTA GAG CCG GCA ACA ATG CAA GGC TAC GAC CGT GAC CCA CGT TCT ATC GCA AAG CGC GCT GAA GAC TTC ATG CGC TCT ACT GGC
  91   D   I   L   E   P   A   T   M   Q   G   Y   D   R   D   P   R   S   I   A   K   R   A   E   D   F   M   R   S   T   G

 361  GTT GCA GAT ACT GTA CTT ATC GGT CCT GAG CCA GAA TTC TTC CTA TTT GAC GAC GTG AAA TTC GCG ACT GAC ATG TCA GGC TCT TTC TTC
 121   V   A   D   T   V   L   I   G   P   E   P   E   F   F   L   F   D   D   V   K   F   A   T   D   M   S   G   S   F   F

 451  AAG ATC GAT GAC GTA GAA GCA GCA TGG AAC ACA GGT TCT GAT TAC GAA GAA GGT AAC AAA GGT CAC CGT CCA GGC GTT AAA GGT GGT TAC
 151   K   I   D   D   V   E   A   A   W   N   T   G   S   D   Y   E   E   G   N   K   G   H   R   P   G   V   K   G   G   Y

 541  TTC CCT GTA GCT CCA GTG GAT TCA TCT CAA GAC ATC CGT TCT GCT ATG TGT CTA GTA ATG GAA GAA ATG GGT CTT GTT GTT GAA GCG CAC
 181   F   P   V   A   P   V   D   S   S   Q   D   I   R   S   A   M   C   L   V   M   E   E   M   G   L   V   V   E   A   H

 631  CAC CAC GAA GCA ACA GCG GGT CAA AAC GAA ATC GCA ACT CGT TTC AAC ACG CTA ACA ACC AAA GCT GAC GAA ATC CAA ATC TAC AAG TAC
 211   H   H   E   A   T   A   G   Q   N   E   I   A   T   R   F   N   T   L   T   T   K   A   D   E   I   Q   I   Y   K   Y

                                                                                                                 -------------
 721  GTT GTA CAC AAC GTT GCT CAC GCG TTT GGT AAA ACG GCA ACA TTC ATG CCT AAA CCA CTT GTT GGT GAC AAC GGT AGC GGT ATG CAC GTT
 241   V   V   H   N   V   A   H   A   F   G   K   T   A   T   F   M   P   K   P   L   V   G   D   N   G   S   G   M   H   V
                                                                                                                 -------------
      -------
 811  CAC CAA TCT CTA GCG AAA GAT GGT GTA AAC CTA TTT GCT GGT GAC AAG TAC GGC GGT CTA TCT GAA ATG GCG CTT TAC TAC ATT GGC GGT
 271   H   Q   S   L   A   K   D   G   V   N   L   F   A   G   D   K   Y   G   G   L   S   E   M   A   L   Y   Y   I   G   G
      -------

 901  ATC ATC AAA CAC GCT CGT GCA ATC AAC GCA TTT GCT AAC CCA TCA ACA AAC TCG TAC AAA CGT CTT GTA CCA GGC TTC GAA GCG CCA GTT
 301   I   I   K   H   A   R   A   I   N   A   F   A   N   P   S   T   N   S   Y   K   R   L   V   P   G   F   E   A   P   V

 991  ATG CTT GCT TAC TCT GCA CGT AAC CGC TCT GCT TCA ATC CGT ATC CCT GTG GTA CCA AGC CCG AAA GCA CGT CGT ATC GAA GTT CGC TTT
 331   M   L   A   Y   S   A   R   N   R   S   A   S   I   R   I   P   V   V   P   S   P   K   A   R   R   I   E   V   R   F

1 081 GGT GAC CCA GCG GCT AAC CCA TAC CTA TGC TTT GCA TCA ATG CTA ATG GCT GGT CTT GAC GGT ATT AAG AAC AAG ATC CAC CCA GGC GAA
 361   G   D   P   A   A   N   P   Y   L   C   F   A   S   M   L   M   A   G   L   D   G   I   K   N   K   I   H   P   G   E

                                AMP
                                ...
1 171 GCA ATG GAT AAA GAC CTT TAC GAT CTA CCA GCA GAA GAA TCA GCA GAA ATC CCA ACG GTT GCA TAC TCA CTG AAA GAC GCA CTA GCA GAG
 391   A   M   D   K   D   L   Y   D   L   P   A   E   E   S   A   E   I   P   T   V   A   T   S   L   K   D   A   L   A   E

1 261 CTA GAT GCT GAC CGT GAA TTC CTA ACA GCG GGT GGT GTA TTC TCT GAC GAC TTC ATC GAC TCT TAC ATC GAG CTA AAA TCT CAG GAC GTA
 421   L   D   A   D   R   E   F   L   T   A   G   G   V   F   S   D   D   F   I   D   S   Y   I   E   L   K   S   Q   D   V

1 351 GAG CGC GTG AAC ATG ACA ACT CAC CCA GTT GAG TTC GAA CTT TAC TAC TCT GTA TAA
 451   E   R   V   N   M   T   T   H   P   V   E   F   E   L   Y   Y   S   V   *
```

Fig. 4.5. Nucleotide sequence of the V. alginolyticus glnA gene. The derived amino acid sequence is given in the one-letter code from positions 1 to 1407 (469 residues). The direct (boldface type) and indirect (underlined) protein alignment with the E. coli GS, the AMP binding site (•••) and the amino acid sequence containing the oxidizable histidine of GS (boxed) are shown (Miranda-Rios et al.,

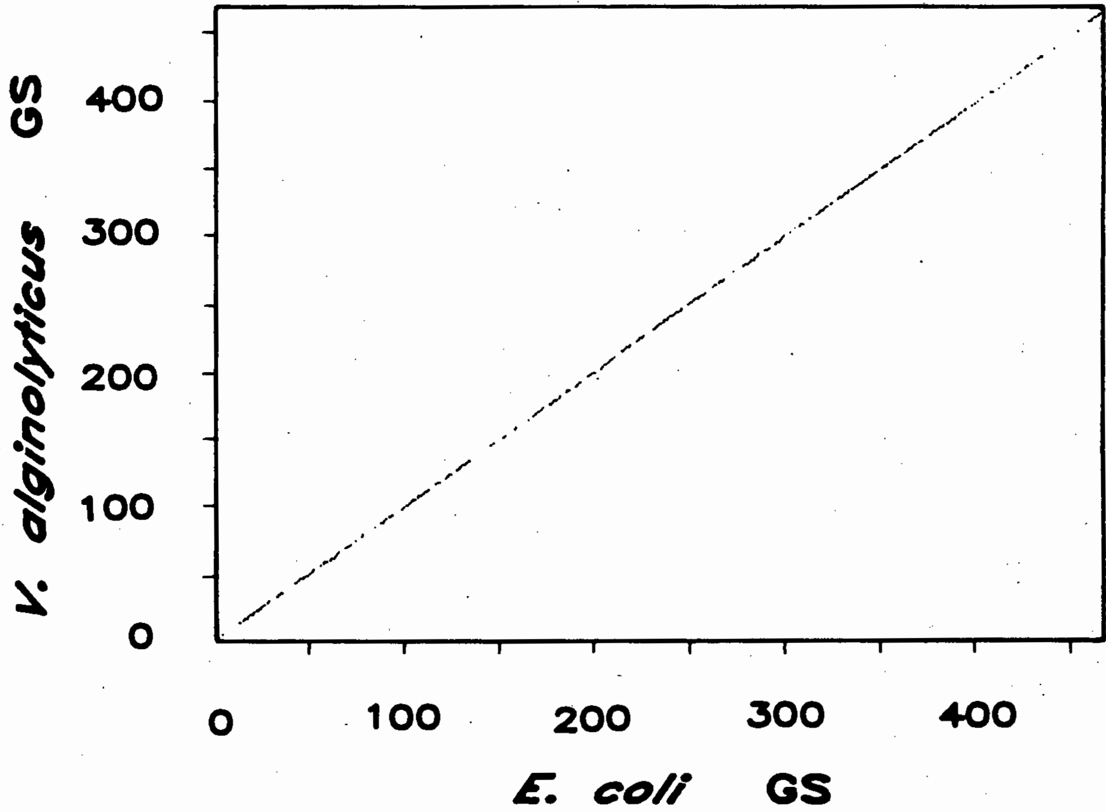
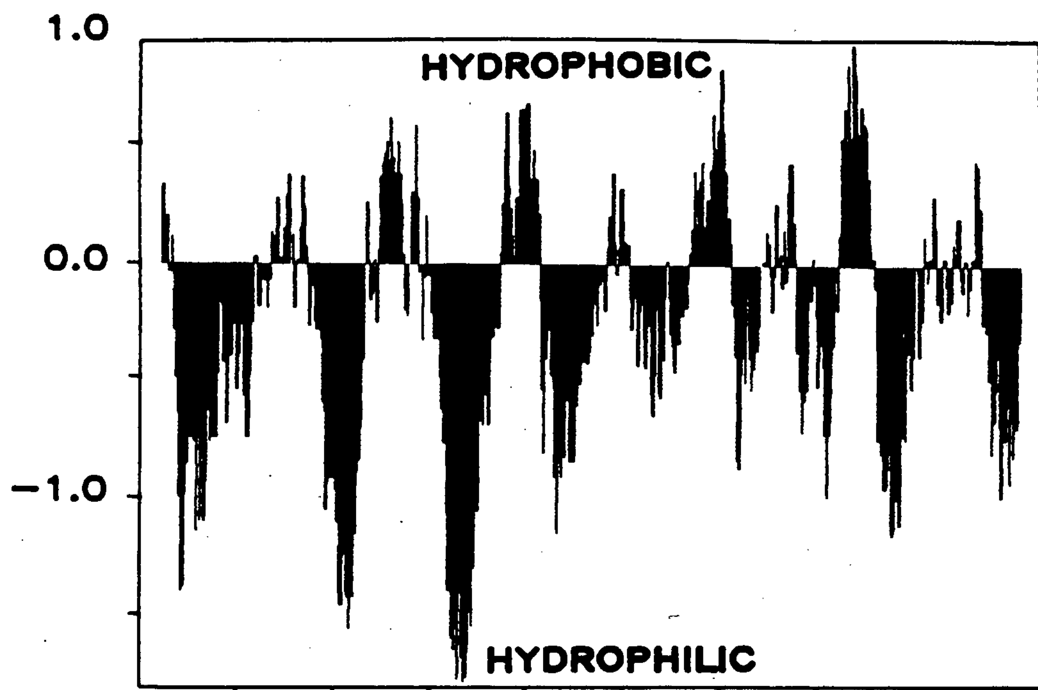


Fig. 4.6. Alignment of deduced amino acid sequences of GS enzymes from E. coli (Rocha et al., 1985; Colombo and Villafranca, 1986; Miranda-Rios et al., 1987) and V. alginolyticus.

Fig. 4.7 shows the plots of hydropathies of GS from V. alginolyticus and E. coli. The profiles of these plots did not differ significantly and lack long stretches of hydrophobic and hydrophilic amino acids which is a characteristic of soluble-globular proteins (Miranda-Rios et al., 1987). There is a notable difference in hydropathy between amino acid residues 60-90 in both sequences. This region is slightly hydrophobic in the V. alginolyticus GS and highly hydrophobic in E. coli. The carboxy terminus of the E. coli enzyme has longer stretches of hydrophilic residues around amino acids 400-440. In addition, a slightly hydrophobic region present around residue 250 in the V. alginolyticus GS is absent in the E. coli GS. Alpha helix potential profiles of the deduced amino acid sequences of V. alginolyticus and E. coli GS genes are given in Fig. 4.8. Most of the peaks in the two profiles occur at corresponding positions indicating a strong conservation of secondary protein structure.

Bodasing et al. (1985) have shown that the wild-type V. alginolyticus GS can be regulated by an adenylylation-deadenylylation system which is typical of all Gram-negative bacteria. The amino acid sequence around the E. coli Tyr residue 398 was of interest because it has been shown to be the residue which is adenylylated under conditions of abundant glutamine (Shapiro and Stadtman, 1968). Tyr^{397} in V. alginolyticus was identified as the site of adenylylation (Fig. 4.5). The Tyr target of adenylylation has been identified in GSs from R. leguminosarum (Colonna-Romano et



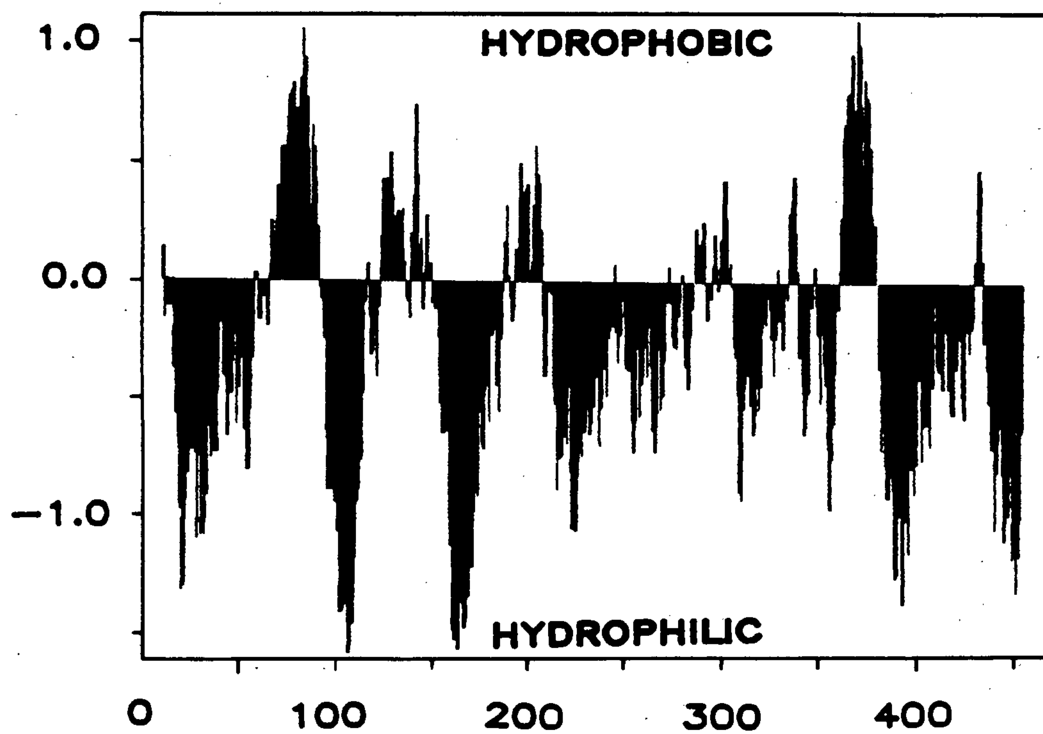
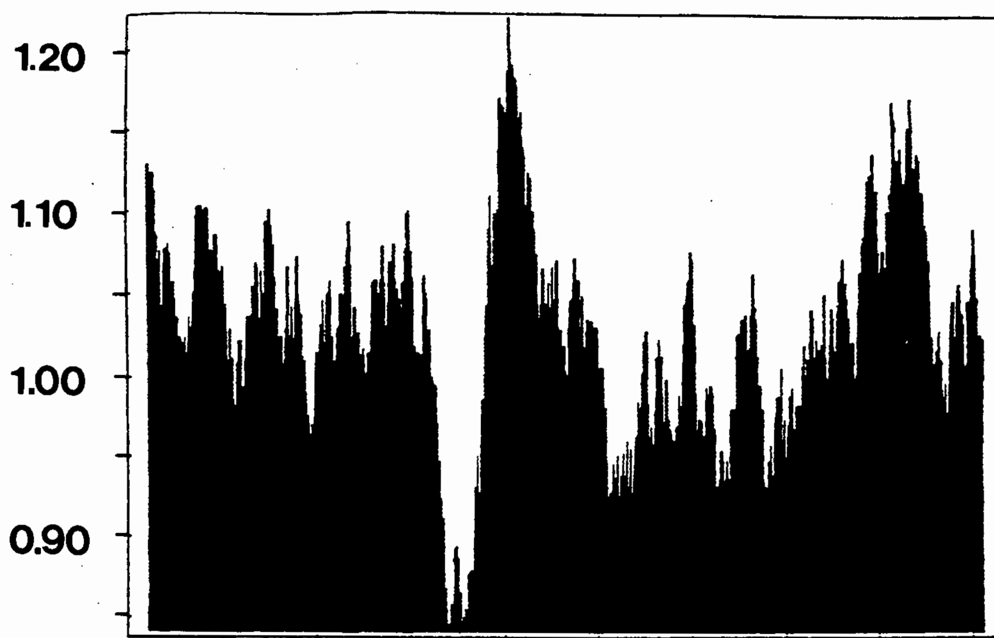


Fig. 4.7. Hydropathy profiles of the deduced amino acid sequences of the *V. alginolyticus* and *E. coli* (Miranda-Rios *et al.*, 1987) GS enzymes.



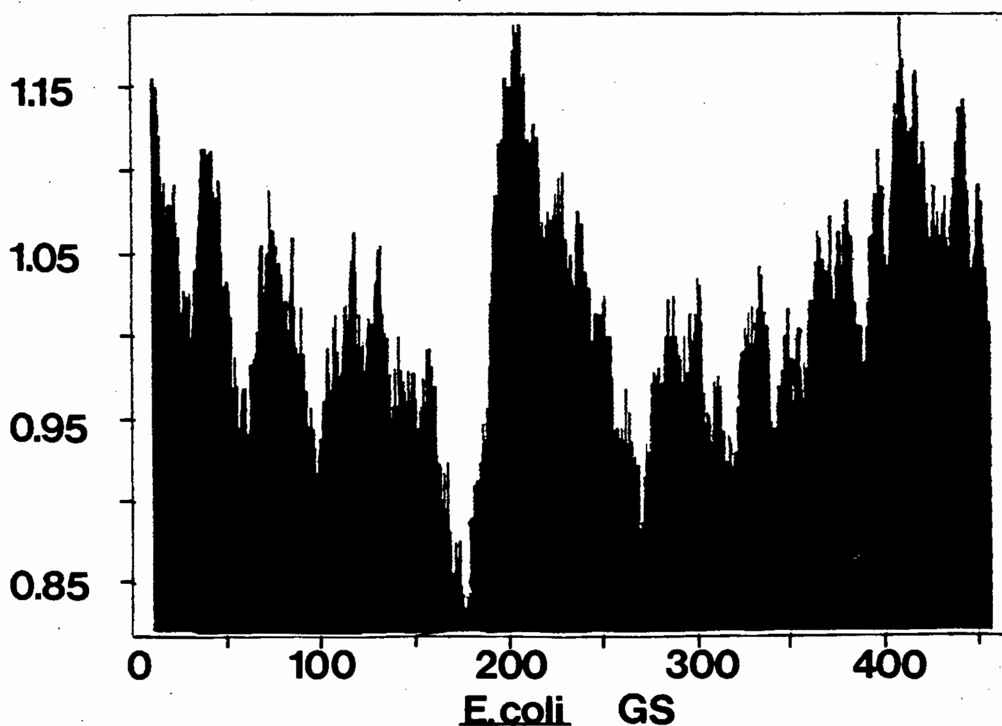


Fig. 4.8. Alpha helix potential profiles of the deduced amino acid sequences of the *V. alginolyticus* and *E. coli* (Miranda-Rios *et al.*, 1987) GS enzymes.

al., 1987), Azospirillum brasilense Sp7 (Bozouklian and Elmerich, 1986), T. ferrooxidans (Rawlings et al., 1987), Anabaena 7120 (Tumer et al., 1983) and S. typhimurium (Janson et al., 1986). The Anabaena GS is unusual in that although it has a Tyr residue at the corresponding position, it is not adenylylated when cloned in E. coli (Fisher et al., 1981). The inability of the Anabaena GS to be adenylylated was thought to be due to the differences in protein sequence adjacent to the Tyr residue (residues 392-400) (Colombo and Villafranca, 1986). Colonna-Romano et al. (1987) analyzed the secondary structure of GSs from R. leguminosarium, E. coli, A. brasilense and Anabaena 7120 in this region and were unable to find a correlation between lack of adenylylation and secondary structure around the target Tyr residue. The precise reason for the lack of adenylylation in Anabaena 7120 is as yet unknown. The C. acetobutylicum and B. subtilils GSs are not subject to adenylylation and lack the target Tyr residue (Janssen et al., 1988; Struach et al., 1988).

A second region of functional significance in E. coli is the sequence **MHCHM** (residues 269-273) which contains a His residue which is oxidized with loss of catalytic activity (Farber and Levine, 1986). The S. typhimurium GS has has the identical sequence at the corresponding position. Studies on the crystallized GS from S. typhimurium (Almassy et al., 1986) indicated that the metal-ligand sequence **MHCHM** formed part of the active site. Loss of catalytic activity upon oxidative modification could be due to alteration of

binding of divalent cations essential for enzyme activity. The active site channel is formed at the junction of the N and C domains of GS. Almassy et al. (1986) proposed that the adenylyl group at Tyr[398] could affect the active site by interacting with N-domain residues thereby restricting the structure or motion of part of the N-domain with respect to the C-domain. It is possible to assign the sequence **MHVHQ** (Fig. 4.5) in V. alginolyticus as a metal-ligand sequence.

A comparison of GS enzymes from prokaryotic and eukaryotic organisms (Rawlings et al., 1987) showed that although the homology between GS enzymes could be as low as 14% the major part of this homology was located in five conserved regions. This comparison has been extended to include R. leguminosarum (Colonna-Romano et al., 1987), A. brasilense Sp7 (Bozouklian and Elmerich, 1986), C. acetobutylicum (Janssen et al., 1988) B. subtilis (Strauch et al., 1988) and V. alginolyticus GS enzymes (Fig. 4.9). An interesting feature of the five conserved regions is that they all form part of the C-domain and are associated with the GS active site (Almassy et al., 1986). The X-ray crystallography studies of Almassy et al. (1986) revealed six active sites in the S. typhimurium GS. Each active site was at the interface between two subunits and related to each other by the 6-fold axis of symmetry. Regions 2 to 5 form β-strands closely associated with the two Mn^{2+} ions of one subunit and region 1 contains the Trp residue which is thought to complete the active site formed between adjacent subunits. Region 3 is of interest because it resembles the amino acid

Fig. 4.9. Comparison of amino acid sequence alignment of five regions of homology of GS enzymes from *R. leguminosarum* (Rl) (Colonna-Romano *et al.*, 1987); *A. brasilense* Sp7 (Ab) (Bozouklian and Elmerich, 1986); *C. acetobutylicum* (Ca) (Janssen *et al.*, 1987); *B. subtilis* (Bs) (Strauch *et al.*, 1988); *B. japonicum* (Bj) (Carlson *et al.*, 1986); *Phaseolus vulgaris* (Pv) (Gebhardt *et al.*, 1986); *alfalfa* (Af) (Tischer *et al.*, 1986); *Chinese hamster* (Ch) (Hayward *et al.*, 1986); *T. ferrooxidans* (Tf) (Rawlings *et al.*, 1987); *Anabaena* 7120 (An) (Tumer *et al.*, 1983); *S. typhimurium* (St) (Janson *et al.*, 1986); *E. coli* (Ec) (Rocha *et al.*, 1985; Colombo and Villafranca, 1986; Miranda-Rios *et al.*, 1987) and *V. alginolyticus*. The amino acids are identified by the single-letter code and the positions of the amino acids in the GS enzymes are indicated. Numbering of residues begins with the start methionine at the N-terminal end. Regions of identical homology are boxed.

Region I

Position	Organism	Sequence
50	Rl	M F D G S S I G G W K A I N E S D M V L M P D T E T V H M D P F F
50	Ab	M F D G S S I A G W K A I N E S D M I L Q P L D T T A V M D P F S
51	Ca	M F D G S S I D G F V R I E E S D M N L R P N L D S F V I F P W R
51	Bs	M F D G S S I E G F V R I E E S D M Y L Y P D L N T P V I F P W T
42	Bj	G F D G S S T Q Q A E G H S - S D C V L K P V A - - V F P D A A R
53	Pv	N Y D G S S T G Q A P G Q D - S E V I I Y P Q A - - I P K D P F R
54	Af	N Y D G S S T G Q A P G E D - S E V I I Y P Q A - - I F K D P F R
61	Ch	N F D G S S T F Q S E G S N - S D M Y L S P V A - - M F R D P F R
51	Tf	A F D G S S I A G W K G I N E S D M I L L P D P D S A V L D P F M
50	An	P F D G S S I R G W K A I N E S D M T M V L D P N T A W I D P F M
50	St	M F D G S S I G G W K G I N E S D M V L M P D A S T A V I D P F F
49	Ec	M F D G S S I G G W K G I N E S D M V L M P D A S T A V I D P F F
49	Va	M F D G S S V A G W K G I N E S D M V M M P D A S S A V L D P F T

Region II

Position	Organism	Sequence
212	Rl	H E V A A A - Q H E L G I
213	Ab	H E V A A S - Q H E L G I
187	Ca	H E V A E - G Q N E I D F
188	Bs	H E V A P - G Q H E I D F
171	Bj	A E V A K - G Q W E F Q I
190	Pv	G E V M P - G Q W E F Q V
191	Af	G E V M P - G Q W E F Q V
195	Ch	A E V M P - A Q W E F Q I
121	Tf	H E V A T A G Q H E I G V
215	An	H E V A T G G Q C E L G F
215	St	H E V A T A G Q N E V A T
212	Ec	H E V A T A G Q L E V A T
212	Va	H E - A T A G Q N E I A T

Region III

Position	Organism	Sequence
254	Rl	T F M P K P I F G D - - - N G S G M H V H Q S I
255	Ab	T F M P K P V F G D - - - N G S G M H M H Q S I
229	Ca	S F M P K P I F G I - - - N G S G M H V N M S L
230	Bs	T F M P K P L F G V - - - N G S G M H C N L S L
213	Bj	E F H C K P L - G D T D W N G S G M H A N F S T
232	Pv	S F D P K P I K G D - - W N G A G A H T N Y S T
233	Af	S F D P K P I K G D - - W N G A G A H T N Y S T
238	Ch	T F D P K P I P G N - - W N G A G C H T N F S T
254	Tf	T F M P K P V V G D - - - N G S G M H V H Q S L
257	An	T F M P K P I F G D - - - N G S G M H C H Q S L
258	St	T F M P K P M F G D - - - N G S G M H C H M S L
254	Ec	T F M P K P M F G D - - - N G S G M H C H M S L
254	Va	T F M P K P L V G D - - - N G S G M H V H Q S L

Region IV

Position	Organism	Sequence
337	Rl	R N R S A S C R I P
348	Ab	R N R S A S C R I P
313	Ca	K N R T A L I R V P
314	Bs	G N R S P L I A I P
260	Bj	A D R G A S I R V P
308	Pv	A N R G A S I R V G
309	Af	A N R G A S I R V G
317	Ch	A N R S A S I R I P
336	Tf	K N R S A S I R I P
341	An	G N R S A S I R I P
341	St	R N R S A S I R I P
339	Ec	R N R S A S I R I P
337	Va	R N R S A S I R I P

Region V

Position	Organism	Sequence
357	Rl	V E V R F P D P T A N P Y L A F A
357	Ab	V E V R F P D P S A N P Y L A F A
331	Ca	V E L R C P D P S S N P Y L V L A
332	Bs	V E V R S V D P A A N P Y L A L S
281	Bj	L E D R R P N S Q G D P Y Q I V R
328	Pv	F E D R R P A S N M D P Y V V T S
329	Af	F E D R R P S S N M D P Y V V T S
338	Ch	F E D R R P S A N C D P F A V T E
356	Tf	I E V R F P D S T A N P Y L A F S
361	An	L E V R C P D A T S N P Y L A F S
361	St	I E V R F P D P A A N P Y L C F A
358	Ec	I E V R F P D P A A N P Y L C F A
356	Va	I E V R F G D P A A N P Y L C F A

sequence **KXXXXGXXGXGKT** found in several ATP binding proteins (Walker et al., 1982). Region 4 is a probable glutamate binding site. This region is similar to the sequence **DRGASIV** of bovine and chicken GDH which is found close to Lys^{27} which has been implicated in substrate binding in GDH (Moon and Smith, 1973; Rasched et al., 1974; Tischer et al., 1986). The V. alginolyticus GS differed from all other GS enzymes in that it lacked a Val residue in region 2. Repeated sequencing of this region in both directions, using several independent subclones, confirmed the absence of a Val residue at this position.

4.3.3 Analysis of the V. alginolyticus glnA promoter region. The complete nucleotide sequence of the region located upstream of the V. alginolyticus glnA structural gene in pRM210 was determined employing the strategy outlined in Fig. 4.3. The presumptive start codon (ATG) was located 207 bp downstream of the cloning site (Fig. 4.3, Fig. 4.10). A consensus Shine-Delgarno (SD) sequence, two putative NR1 binding sites (Ames and Nikaido, 1985) and a sequence that shared considerable homology with Ntr and Nif promoters (Ausubel, 1984) were identified. Transcription of the structural gene for GS has been shown to be controlled by tandem promoters in a number of organisms (Tumer et al., 1983; Dixon, 1984; Reitzer and Magasanik, 1985; Hirschman et al., 1985). In E. coli the nucleotide sequence of the upstream promoter (glnAp1) resembles the consensus sequence for E. coli promoters and serves to maintain basal levels of GS under conditions of carbon-limitation and nitrogen excess

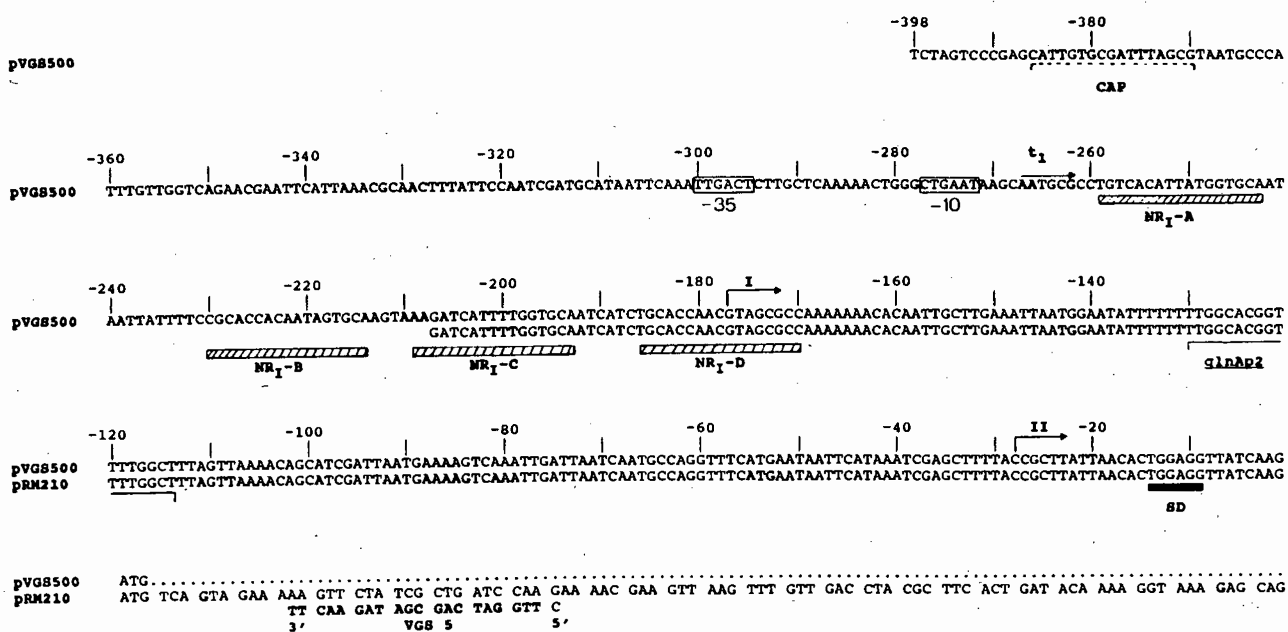


Fig. 4.10. Nucleotide sequence of the promoter region of the V. alginolyticus glnA gene. The solid bar indicates a possible SD sequence and the dashed bars indicate putative NR_I binding sites. The transcriptional start for an upstream promoter is designated t_1 and the -10 and -35 regions are boxed. The downstream promoter (glnAp2) is underlined. The dashed line below the sequence indicates a possible CAP binding site. The end points of deletions in the promoter region are indicated by numbered arrows. The position of primer VGS5 is indicated. The coordinate +1 is defined as the first nucleotide of the glnA structural gene.

(Reitzer and Magasanik, 1985). The downstream promoter (glnAp2) shows homology with the Ntr and Nif promoters. Activation of transcription of GS from glnAp2 occurs when cells are grown in a nitrogen limited environment. Five NR_I binding sites have been identified in the E. coli (Reitzer and Magasanik, 1986) and S. typhimurium (Hirschman et al., 1985) glnA promoter-regulatory region. Site 1 overlaps the portion of the upstream promotor homologous to the canonical **TTGACA** sequence and site 2 overlaps the transcriptional start site (t_1).

Failure to identify an upstream promoter for the V. alginolyticus glnA gene led to the assumption that pRM210 did not contain the entire glnA promoter-regulatory region. A V. alginolyticus prot-T1 glnA gene was isolated from a genomic library constructed in pEcoR251 employing the strategy discussed in Chapter 2 (Rumbak, 1987). The prot-T1 glnA gene was cloned on a 3.76 kb insert in pVGS500 (Fig. 4.2). Restriction enzyme analysis of plasmid pVGS500 showed that the fragment of V. alginolyticus genomic DNA carrying the glnA gene was cloned in the identical orientation in both pRM210 and pVGS500 (Rumbak, 1987). There were no apparent differences in the glnA structural gene as judged by restriction enzyme analysis and DNA hybridization. There were however two important differences between the two glnA clones. The EcoRI-PstI restriction endonuclease fragment containing the glnA promoter region (Fig. 4.3) was approximately 0.5 kb larger in pVGS500 in comparison with pRM210 (Fig. 4.2). In addition, the HindIII-HindIII

restriction endonuclease fragment located downstream of the V. alginolyticus glnA gene (Fig. 4.3) was approximately 2.0 kb smaller in pVGS500 (Fig. 4.2). The 3.27 kb HindIII restriction enzyme fragment apparently contained the regulatory region controlling nitrogen regulated expression of the V. alginolyticus glnA gene in pRM210 (Chapter 3), this downstream region was thus truncated in pVGS500.

S1 nuclease mapping was employed to investigate the possible occurrence of a glnA upstream promoter in pVGS500. This involved the construction of GSP (Fig. 4.2) which is an M13 subclone of pVGS500 carrying the non-coding strand of the V. alginolyticus GS promoter region. The synthetic DNA primer VGS5 (Fig. 4.10) was used for primed synthesis of a strand-specific probe for S1 nuclease mapping and for DNA sequence determination of the V. alginolyticus glnA promoter region in GSP as described in section 4.2. A single protected RNA fragment was detected in RNA extracted from E. coli YMC11(pVGS500) grown under conditions of nitrogen excess (Fig. 4.11). The transcript apparently began with one of two adenine residues designated t_1 in Fig. 4.10. RNA from E. coli YMC11 glnA$^-$, glnL$^-$, glnG$^-$ was not protected by the probe.

4.3.4 Complete nucleotide sequence of the V. alginolyticus glnA promoter-operator region. The complete nucleotide sequence of the V. alginolyticus glnA promoter region is diagramed in Fig. 4.10. The nucleotide sequence of the promoter regions of pRM210 and pVGS500 were compared. The

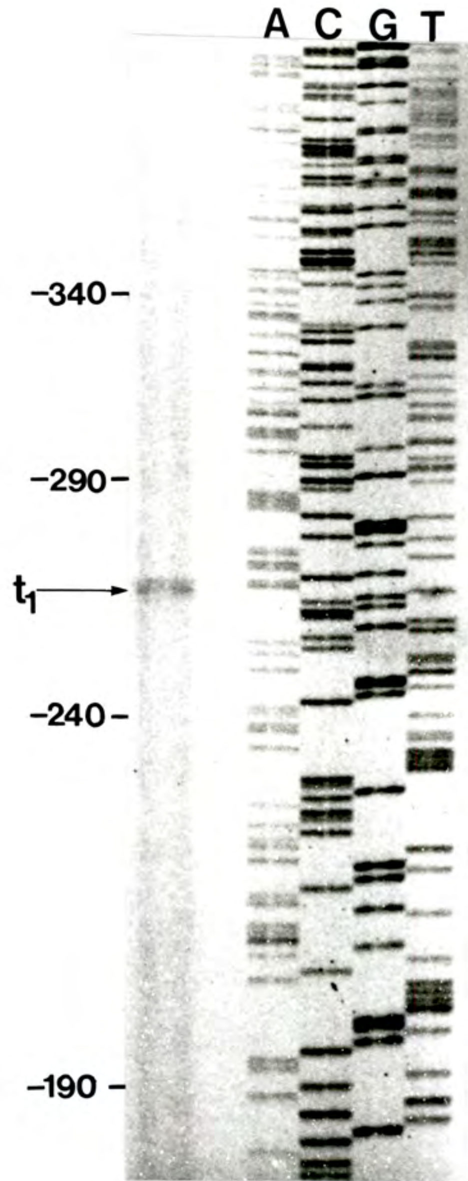


Fig. 4.11. S1 nuclease mapping of the 5′ end of a V. alginolyticus glnA transcript. A uniformly labelled DNA probe was hybridized with RNA extracted from YMC11(pVGS500), treated with S1 nuclease and analyzed on a DNA sequencing gel (6% (w/v)) as described in the methods section. The nucleotide sequence products of GSP DNA (A,C,G,T) were used as M_r markers. The transcriptional start of the glnAp1 promoter (t_1) is indicated bu an arrow.

glnA promoter region in pVGS500 was found to span an area of approximately 400 bp. This supported the assumption that the entire glnA promoter region was not present in pRM210. The nucleotide sequence of the region which was common to both pVGS500 and pRM210 was identical. Analysis of the V. alginolyticus glnA region revealed considerable similarity with the E. coli glnA promoter region (Reitzer and Magasanik, 1985; 1986). The glnA gene of V. alginolyticus is apparently transcribed from tandem promoters. The nomenclature of Reitzer and Magasanik (1985) has been adopted for naming of the tandem promoters. The upstream promoter (glnAp1) starts at position -267, where the coordinate +1 is defined as the first nucleotide of the glnA structural gene. A comparison of the V. alginolyticus and E. coli glnAp1 promoters is shown below.

	-35		-10
σ^{70} consensus	**TTGACA**	**-N_{15-19}**	**-TATAAT**
V. alginolyticus	**TTGACT**	**-N_{17}**	**-CTGAAT**
E. coli	**TTGCAC**	**-N_{18}**	**-TTCCAT**

The -10 and -35 RNA polymerase contact sites in E. coli are 50% homologous to the consensus sequences and are separated by one more nucleotide than is optimal. The best -10 RNA polymerase contact site in V. alginolyticus is 50% homologous to the consensus sequence. In contrast the -35 RNA polymerase contact site is 83% homologous to the consensus sequence. The divergent -10 RNA polymerase contact site suggests that the V. alginolyticus glnAp1 promoter is relatively weak. The final T of this site, considered to be essential, is however conserved. This is

supported by the fact that the large quantity of RNA (200μg) used in S1 mapping experiment produced a relatively weak signal. Activation of transcription from the *glnAp1* promoter of *E. coli* has an absolute requirement for the catabolite activator protein bound to cAMP (Reitzer and Magasanik, 1985). A nucleotide sequence which shares considerable homology with the catabolite activating protein recognition sequence (Ebright, 1982) is located between positions -386 and -387 in the *V. alginolyticus glnA* promoter region:

V. alginolyticus	**C A N T G T G C N N T N N N N C G**
consensus sequence	**A A N T G T G A N N T N N N N C A**
E. coli	**C T N T G T G A N N G N N N N C A**

Whether or not the *V. alginolyticus glnAp1* has a requirement for the catabolite activator protein remains to be established.

The presumptive *glnAp2* promoter of *V. alginolyticus* is located 137 bases downstream from t_1 and extends from positions -130 to -114. This promoter which was present in both pRM210 and pVGS500 shows homology with other promoters that are activated during nitrogen-limited growth (Ausubel, 1984):

	-24 -12
consensus sequence	CT**GG**CACNNNNNTT**GC**A
V. alginolyticus	TT**GG**CACNNNNNTG**GC**T
E. coli	TT**GG**CACNNNNNTC**GC**T

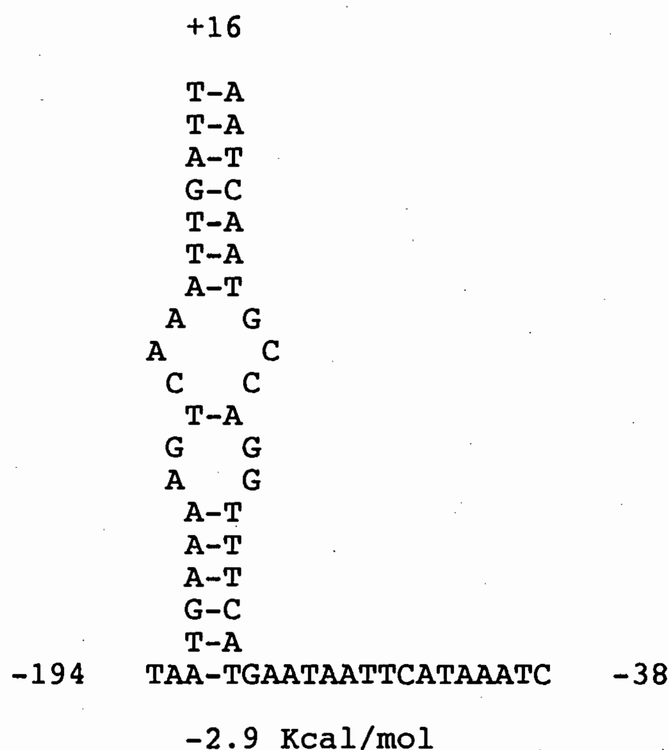
Reitzer and Magasanik (1985) have shown that expression from the *E. coli glnAp2* promoter required the products of the

glnG and glnF genes. Full expression from this promoter requires growth in a nitrogen-limited environment. S1 mapping analysis indicated that expression of GS from pVGS500 in an E. coli $glnA^-$, $glnL^-$, $glnG^-$ strain was initiated at the upstream promoter (Fig. 4.11). It is probable that full expression from the presumptive V. alginolyticus glnAp2 promoter has a requirement for the products of the glnG and glnF genes as is the case for E. coli. Deletion analysis of the promoter region of pRM211, a deletion derivative of pRM210, suggested that initiation of transcription of glnA from glnAp2 did occur. Deletion of the glnAp2 promoter in pRM211 resulted in a $GlnA^-$ phenotype (designated II in Fig. 4.10) while the deletion with an end point 50 bp upstream from glnAp2 (designated I in Fig. 4.10) had a $GlnA^+$ phenotype.

Four putative NR_I binding sites are located between the V. alginolyticus glnAp1 and glnAp2 promoters. These binding sites share between 82 and 70% homology with the NR_I consensus sequence (Table 4.4). In E. coli binding of NR_I at the strong binding sites (sites 1 and 2) activates initiation of expression from glnAp2 (Reitzer and Magasanik, 1986). As these sites overlap the upstream promoter, simultaneous repression of the glnAp1 promoter results from simple obstruction. The NR_I binding sites in the V. alginolyticus glnA promoter region do not overlap the promoter region.

Table 4.4. DNA sequences of putative NR_I binding sites in the V. alginolyticus glnA promoter region.

Binding site	DNA sequence	% Homology with NR_I consensus sequence
NR_I consensus sequence	AAAA TGCACC TGGTGCA TTTT	
NR_I-A	TGTCACATTATGGTGCA	82
NR_I-B	CGCACCACAATAGTGCA	88
NR_I-C	AAGATCATTTTGGTGCA	76
NR_I-D	TGCACCAACGTAGCGCC	70



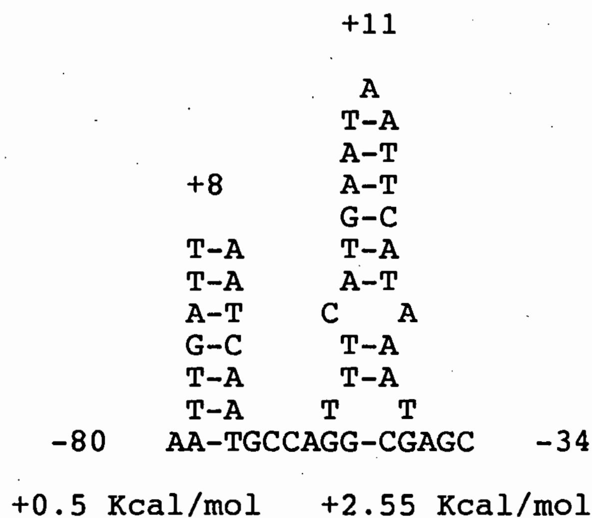


Fig. 4.12. Possible secondary structures in the <u>V. alginolyticus</u> <u>glnA</u> promoter region. Numbering of coordinates is as in Fig.4.10.

An interesting and unique feature of the V. alginolyticus glnA promoter region is a sequence with dyad symmetry between glnAp2 and the translational start. This sequence, which has the potential to form stem-loop secondary structures (Fig. 4.12) may be involved in the regulation of V. alginolyticus GS by oxygen and/or temperature.

4.3.5 Nucleotide sequence of the V. alginolyticus glnL gene. Situated approximately 760 bp downstream of the V. alginolyticus glnA gene is an ORF encoding a polypeptide of 347 amino acid residues (Fig. 4.13). The open reading frame is preceded by a consensus Shine-Delgarno sequence (**AGGACG**) 4 bp upstream from the presumptive start codon (**GTG**) which codes for a Val residue. The amino acid sequence of the protein shares approximately 53% homology with the E. coli glnL product (NR_{II}) (Fig. 4.13, Fig. 4.14). Hydropathy profiles of the deduced amino acid sequences of the two proteins (Fig. 4.15) are very similar. Most of the peaks and troughs appear to occur at corresponding positions in the two proteins. The V. alginolyticus polypeptide however appears to have a region extending from amino acid residues 80-100 which is more hydrophobic. Alpha helix potential profiles of the two proteins indicates that the major difference in the secondary structures of the two proteins occurs in the central region. The N- and C- termini of the two proteins do not differ significantly (Fig. 4.16). Situated 42 bp downstream of the stop codons for the presumptive V. alginolyticus glnL gene, is a second ORF encoding part of a polypeptide (27 amino acids) (Fig.4.13).

```
                                                  GGTCGACACTGTGTAAAATGTAAACACAGCAACAAAA

-720   TATTAGGCTCGCTCGCAGGCCTTTTTTGTATTCGTCAGTCAAGATTCGACGTTTAAC ATGAGGGGATAATAAAAATACGCTTGGACTTTTTACCTTGTAGGTCAAAGAGCTAAGCCATTG

-600   AAACGTGAAATCATCGATCAAAAGAAGGTGAGCAATGAAAGTTCAGCTTATTCCATC GCTATGTGCTCTCAGCGGATTGTTAATCGCCTCCATCGCATCAGCGCAGGTTGCTTATACATG

-480   GGTAGATAAAAATGGCGGTGGTTCATTTTAGTGATACGCCAAACCAAGGTGCAAAAG CCATGCCGCTGCCAAACCTTGAAGCCAGGCACCCGCTCCTAAGGTAGAAAGCACGGAATCACT

-360   CGCCCCTCAGACCAAGCCATCGAAAGAGCCACAAGACAAGCCGGAAAAGAAAACCGATAAACCTCTCCCATTAAAGCTCTCGATGCTGACTCCTCAACATAACGAAACCATCCGCAGCAA

-240   CCGAGGCATCATCAATATTCAACTGGAACCAAGCTTGCATCTACTAGCCCTATAACGGTGCATTTACATAGAGCAAGTGTTAAGCCGACCGTAAAAGGGGCAAAATGAAATATCTGGTGA

-120   AAAAAATAGCATCACYGCGGTGCTTTTCACTTTTTCTTCTGAGATTTATTCGCCATACTTAACGTTCAATTGCACCATTATTGTGCAACGCACAATCTTAAAGCAAAACAAGGACGTAAT
                                                                                  NR_I                                       SD

1      GTG GAT ACC AGT CTT CCT AGT GCC ATT CTC AAC AAC ATG GTG ACA GCA ACG CTG ATT CTT GAT GAT GGA TTG GCT ATC CGT TAT GCC AAT
1       V   D   T   S   L   P   S   A   I   L   N   N   M   V   T   A   T   L   I   L   D   D   G   L   A   I   R   Y   A   N

91     CCG GCT GCC GAG TTG CTG TTT TCT CAA AGC GCG AAA CGT ATT GTT GAA CAA TCG CTT TCT CAA TTG ATT CAA CAT GCG TCG CTG GAT CTC
16      P   A   A   E   L   L   F   S   Q   S   A   K   R   I   V   E   Q   S   L   S   Q   L   I   Q   H   A   S   L   D   L

181    GCG CTG CTC ACT CAA CCA TTG CAA AGT GGC CAA AGC ATC ACC GAC AGT GAC GTC ACT TTC GTT GTT GAT GGC CGC CCC TTA ATG CTA GAG
31      A   L   L   T   Q   P   L   Q   S   G   Q   S   I   T   D   S   D   V   T   F   V   V   D   G   R   P   L   M   L   E

271    GTG ACA GTT AGC CCA ATC ACT TGG CAA AAG CAG TTG ATG CTT CTG GTT GAG ATG CGC AAA ATT GAT CAG CAG CGC CGA CTC TCT CAA GAA
46      V   T   V   S   P   I   T   W   Q   K   Q   L   M   L   L   V   E   M   R   K   I   D   Q   Q   R   R   L   S   Q   E

361    CTG AAC CAG CAC GCC CAA CAA CAA GCG GCG AAA CTG TTA GTC CGA GGG CTT GCA CAT GAG ATC AAA AAC CCG CTA GGT GGA TTA CGT GGT
61      L   N   Q   H   A   Q   Q   Q   A   A   K   L   L   V   R   G   L   A   H   E   I   K   N   P   L   G   G   L   R   G

451    GCA GCG CAA TTG TTA GAA AAA ATG CTG CCA GAT CCC TCT CTC ACC GAG TAT ACG CAC ATC ATT ATT GAG CAA GCC GAC CGT TTA AGA GCG
76      A   A   Q   L   L   E   K   M   L   P   D   P   S   L   T   E   Y   T   H   I   I   I   E   Q   A   D   R   L   R   A

541    CTT GTT GAT CGA TTA CTC GGA CCA CAA AAG CCA GGT AAG AAG ACA CAA GAA AAC TTG CAT CAA ATT CTG GAA AAG GTC CGC CAG TTG GTT
91      L   V   D   R   L   L   G   P   Q   K   P   G   K   K   T   Q   E   N   L   H   Q   I   L   E   K   V   R   Q   L   V

631    GAA CTA GAA TCG CAA CGT TCA ATC GTG ATT GAA CGT GAT TAC GAC CCG AGC TTG CCA GAA ATT TTG ATG GAT GCG GAC CAA ATC GAA CAG
106     E   L   E   S   Q   R   S   I   V   I   E   R   D   Y   D   P   S   L   P   E   I   L   M   D   A   D   Q   I   E   Q

721    GCG ATG CTT AAT ATC GTC AGT AAT GCA GCG CAA ATT CTT AGC CAT CAA GAA CAC GGT AAA ATC ACC ATT CGC ACC AGA ACG GTG CAT CAA
121     A   M   L   N   I   V   S   N   A   A   Q   I   L   S   H   Q   E   H   G   K   I   T   I   R   T   R   T   V   H   Q

811    GCT AAT ATT CAC GGC AAA CGC TGT AAA CTC GCT GCG CGT GTC GAA ATC ACA GAC AAC GGC CCA GGC ATT CCA CCG GAA TTA CAA GAC ACG
136     A   N   I   H   G   K   R   C   K   L   A   A   R   V   E   I   T   D   N   G   P   G   I   P   P   E   L   Q   D   T

901    CTG TTC TAT CCC ATG GTC AGT GGC CGC GAG [GGA GGA ACT GGC TTA GGG] CTG TCT ATT TCA CAA AAT TTG ATC GAC CAG CAC AAT GGA [AAA]
151     L   F   Y   P   M   V   S   G   R   E  [ G   G   T   G   L   G ] L   S   I   S   Q   N   L   I   D   Q   H   N   G  [ K ]

991    ATA GAC GTA GAA AGC TGG CCT GGT CAC ACA ACA TTT ACT ATT TAT TTG CCG ATC TGA TTG CGC TAA
166     I   D   V   E   S   W   P   G   H   T   T   F   T   I   Y   L   P   I   *   L   R   *

1027   TGGACTTCAAGTCGCATCAGTTTTTCGGTTTGCTGCAAGGATTACTAC ATG AGT AAA GGA TAT GTT TGG GTC GTT GAT GAC GAC AGT TCC ATT CGC TGG GTG
1                                                SD       M   S   K   G   Y   V   W   V   V   D   D   D   S   S   I   R   W   V

1129   ATG GAG AAA ACC TCT CCT CTC CCA ACA
19      M   E   K   T   S   P   L   P   T
```

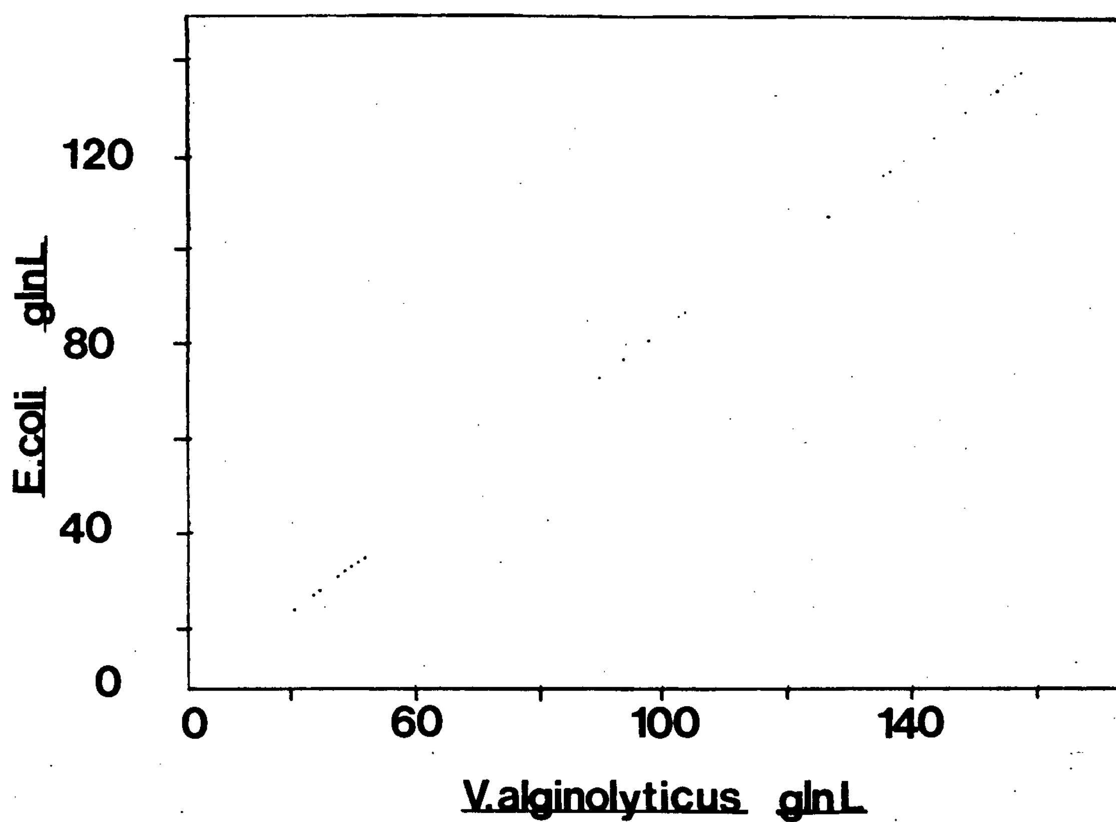
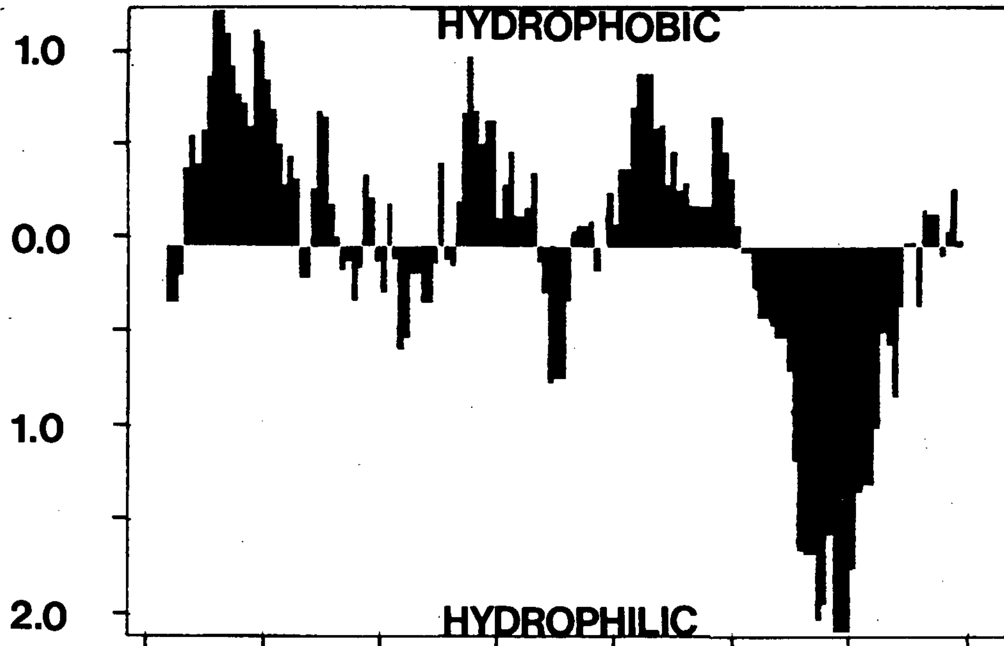


Fig. 4.14. Alignment of the deduced amino acid sequences of the glnL genes from E. coli (Miranda-Rios et al., 1987) and V. alginolyticus.



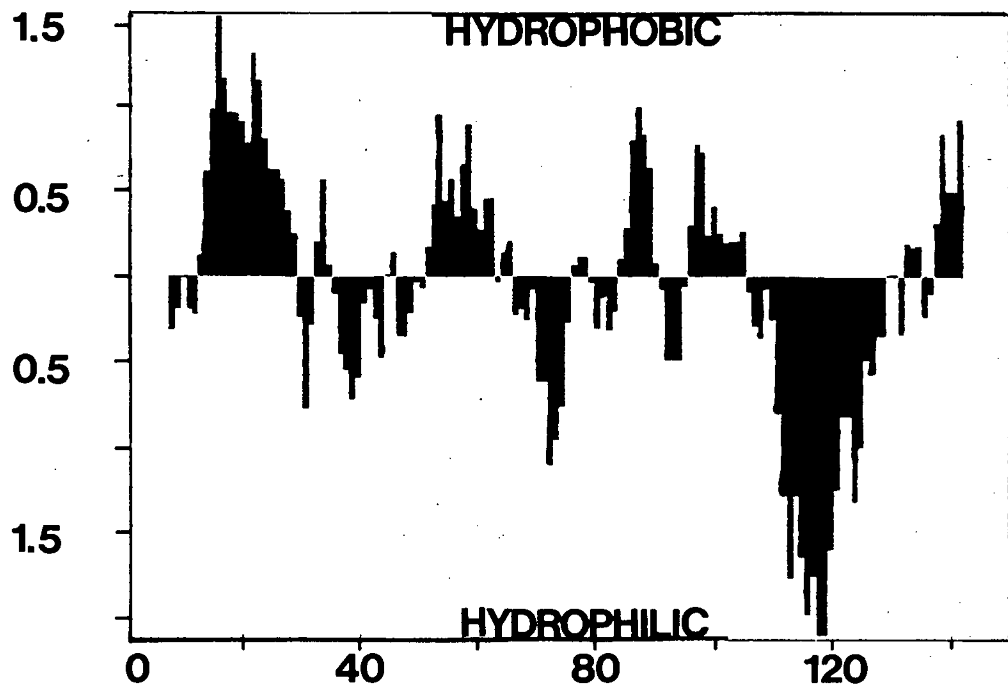
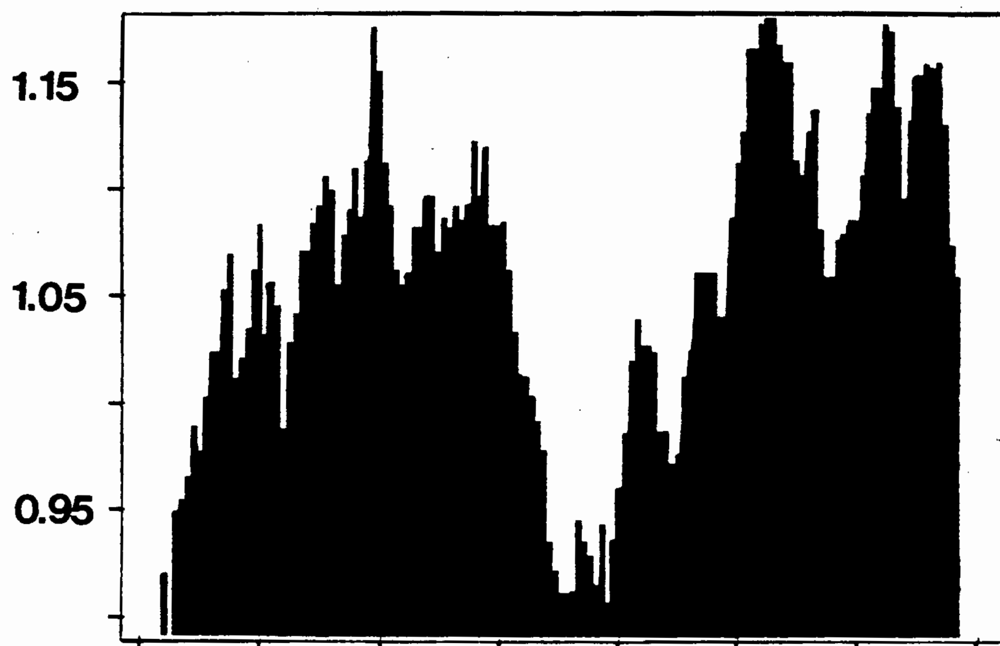


Fig. 4.15. Hydropathy profiles of the deduced amino acid sequences of the V. alginolyticus and E. coli (Miranda-Rios et al., 1987) glnL genes.



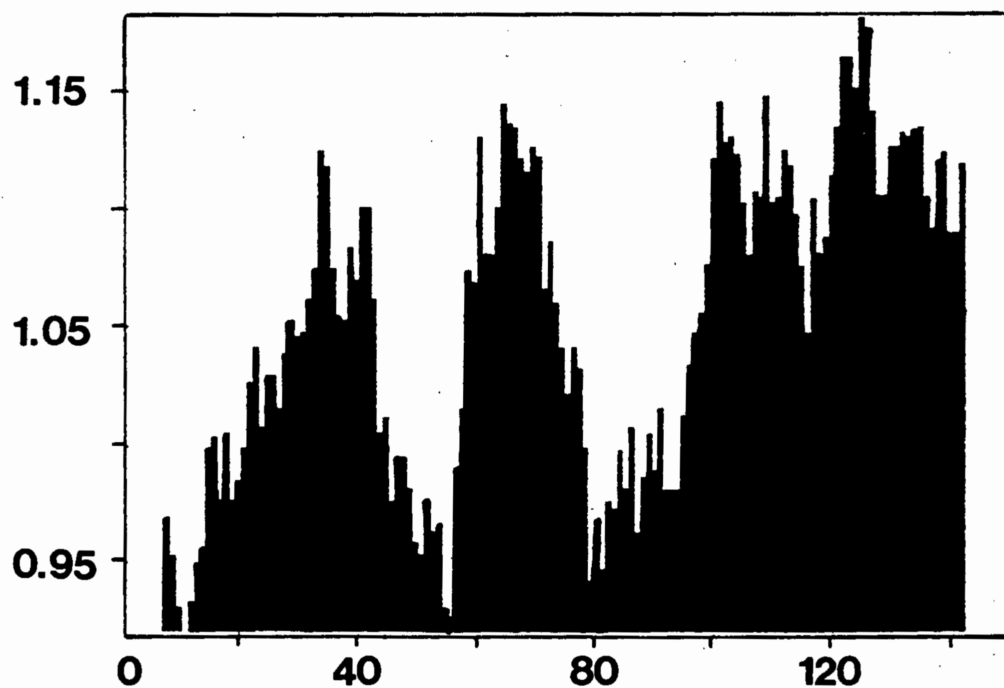


Fig. 4.16. Alpha helix potential profiles of the deduced amino acid sequences of the _V. alginolyticus_ and _E. coli_ (Miranda-Rios _et al_., 1987) _glnL_ genes.

The presumptive start codon (**ATG**) is preceded by a possible Shine-Delgarno sequence (**AGGATT**). The deduced amino acid sequence of the 27 amino acid long polypeptide has 59% homololgy with the amino terminal end of the E. coli glnG product (NR_I).

The glnL and glnG genes of E. coli form part of the complex glnALG operon and are involved in the transcriptional regulation of GS. The regulatory genes are located approximately 250 bp downstream from the glnA structural gene (Ueno-Nishio et al., 1984). The two genes are separated by 11 bp (Miranda-Rios et al., 1986) and are cotranscribed from the glnL promoter or readthrough from the glnA tandem promoters. The V. alginolyticus glnA region has two ORFs located approximately 750 bp downstream from the glnA gene and separated by 42 bp. The complete and partial polypeptides encoded by these ORFs are homologous to the E. coli entire glnL and partial glnG gene products. It seems reasonable therefore that the V. alginolyticus glnA gene, like that of the enteric bacteria, forms part of the glnALG operon.

Activation of transcription of GS from glnAp2 requires a phosphorylated NR_I protein (Hirschman et al., 1985; Reitzer and Magasanik, 1986). Phosphorylation-dephosphorylation of NR_I is catalyzed by the protein kinase NR_{II}. It has been observed that all the protein kinases contain a Gly rich region followed by an invariant Lys residue about 15 bp from the carboxy terminus (Hunter and Cooper, 1985). The amino

acid sequence **GGTGLG** from residues 310 to 315 (Fig. 4.13) in the putative V. alginolyticus glnL gene corresponds to this region. The Lys residue is present 14 bp downstream from the Gly rich region. The V. alginolyticus protein is therefore likely to be functionally analogous to the E. coli NR_{II} protein. A similar Gly rich sequence was observed in a number of nucleotide binding proteins (Wierenga and Hol, 1983). The amino acid sequence was found to form an elbow around the nucleotide making contact with the ribose ring.

The NR_{I} and NR_{II} proteins belong to a family of two-component regulatory systems that are involved in mediating the cellular response to changing environmental conditions (Nixon et al., 1986; Ronson et al., 1987; MacFarlane and Merrick, 1987). One component of each system is thought to act as an environmental sensor that transmits a signal to the second regulatory component which then effects the response. Analysis of the deduced amino acid sequences has indicated that about 200 amino acid residues of the C terminal region are conserved among the proteins that act as sensors. In the regulators, approximately 120 amino acid residues of the N-terminal region are conserved (Ronson et al., 1987). In a comparative analysis of the predicted amino acid sequence of a number of proteins belonging to the sensor class of proteins (Fig. 4.17), MacFarlane and Merrick (1987) were able to identify three regions that were highly conserved. The three regions of homology are also present in the putative V. alginolyticus glnL protein. Table 4.5 lists the corresponding class of regulator proteins.

REGION I

121	Va NR_{II}	LNQHAQQQAAKLLVR--**GLAHEIRNPLGGLRGAAQLL**EKMLPDPSLTEYTHIIIEQ-A-DRLRALVDRLLGPQKPGKKTQENLH------
121	Kp NR_{II}	QLQHAQQQAAKLLVR--**GLAHEIKNPLGGLRGAAQLL**SKALPDPALMEYTKVIIEQ-A-DRLRNLVDRLLGPQHPGMHVTESIH------
125	Bp NRII	QLTHRSAARSVIALAA-**MLAHEIKNPLSGIRGAAQLL**EQQASSEDRLL-TRLICDE-A-DRIVTLVDRMEVFGDDRPVARGPVNIHS--V
247	Ec PhoM	RVKLEGKNYIEQYV-YA**ILTHELKSPLAAIRGAAEIL**REGPPPEVVARFTDNILTONA-RMQA-LVETLLR-QARLENRQEVVLTAVDVA
230	Ec CpxA	VTALERMMTSQQRLLSD-**ISHELRTPLTRLQLGTALL**RRRSGESKELE--RIETEAQRLDSMINDLLVMSRNQQKNALVSETI---KANQ
224	Ec EnvZ	AAGVKQ-LADDRTLLMA**GVSHDLRTPLTRIRLATEMM**SEQDGYLAESIN-KDIEECNA-IIEQFIDYL--RTGQEMPMEMADLN------
194	Ec PhoR	VTQMHQ-LEGARRNFFA**NVSHELRTPLTVLQGYLEMM**NEQPLEGAVREK-ALHTMREQ-TQRMEGLVKQLLTLSKIEAAPTHLLNEKVDV
465	At VirA	RLEHAQRLEAVGTL-AG**GIAHEFNNILGSILGHAELA**QNSVSRTSVTR-RYIDYIISSGDRAM-LIIDQILTLSRKQERMIKPFSV---S

REGION II

201	Va NR_{II}	QILEKVRQLVELESQRSIVIERDYDPSLPEILMDADQ**IEQAMLNIVSNAA**QILSHQEHGITIRTRTVHQ----ANIHGKRCKLAARVE--
	Kp NR_{II}	KVAERVVKLVSMELPDNVKLVRDYDPSLPELPHDPDQ**IEQVLLNIVRNAL**QALGPEGGEITLRTRTAFQ----LTLHGVRYRLAARID--
	Bp NR_{II}	LDHVKRLAQSGFARNVRFIE--DYDPSLPPVLANQDQ**LIQVFLNLVKNAA**EAVADLGTDAEIQLTTAFRPGVRLSVPGKKSRVSLPLEFC
	Ec PhoM	ALFRRVSEARTVQLAEK-KITLHVTPTEVNVAAEPAL**LEQALGNLLDNAI**DF-TPESGCITLSAEVDQEHVTLK----------------
	Ec CpxA	LWSEVLDNAAFEAEQMGRSLTVNFPPGPWPLYGNPNA**LESALENIVRNAL**RYSHT---KIEVGFAVDKDGITIT----------------
	Ec EnvZ	---AVLGEVIAAESGYEREIETALYPGSIEVKMHPLS**IKRAVANMVVNAA**RYGNGWI-KVSSGTEPNR------------------AWFQ
	Ec PhoR	PMMLRVVEREAQTLSQKKQTFTFEIDNGLKVSGNEDQ**LRSAISNLVYNAVN**-HTPEGTHITVRWQRVPHG----------------AEFS
	At VirA	ELVTEIAPLLRMALPPNIELSFRFDQMQSVIEGSPLE**LQQVLINICKNAS**QAMTANGQIDIIISQAFLPVKKILAHGVMPPGDYVLLSIS

REGION III

287	Va NR_{II}	**ITDNGPGIPPELQDTLFYPMVSGREG------GTGLGLSIS**QNLIDQHNGKIDY-ESWPGHTTFTIYLPI
	Kp NR_{II}	**VEDNGPGIPSHLQDTLFYPMVSGREG------GTGLGLSIA**RSLIDQHSGKIEF-TSWPGHTEFSVYLPIRK
	Bp NR_{II}	**VKDNGSGVPEDLLPNLFDPFVT-----TKQT-GSGLGLALV**AKIVGDHGGIIECESQPRKTTFRVLDADVQRRQATRPKQPR
	Ec PhoM	**VLDTGSGIPDYALSRIFERFYSLPRA--NGQKSSGLGLAFV**SEVARLFNGEVTLRNVQEGGVLASLRLHRHFT
	Ec CpxA	**VDDDGPGVSPEDREQIFRPFYRTDEARDRESGGTGLGLAIV**ETAIQQHRGWVKAEDSPLGGLRLVIWLPLYKRS
	Ec EnvZ	**VEDDGPGIAPEQRKHLFQPFVRGDSAR-TIS-GTGLGLAIV**QRIVDNHNGMLELGTSERGGLSIRAWLPVPVTRAQGTTKEG
	Ec PhoR	**VEDNGPGIAPEHIPRLTERFYRVDKARSRQTGGSGLGLAIV**KHAVNHHESRLNIESTVGKGTRFSFVIPERLIAKNSD
	At VirA	**--DNGGGIPEAVLPHIFEPFFST-RARN---GGTGLGLASV**HGHISAFAGYIDVSSTVGHGTRFDIYLPPSSKEPVNPDSFFGRN
	Ec CheA	**LTVSENMSDDEVAMLIFAPGFSTAEQVTDVS-GRGVGMDVV**LRNIQLMGGHVEIQSKQGTGTTIRILLPLTLAILDGMSVRV

Fig. 4.17. Alignment of the amino acid sequence of <u>V. alginolyticus</u>, <u>K. pneumoniae</u> and <u>Bradyrhizobium parasoniae</u> NR_{II} proteins with the sequences of other homologous regulatory proteins in the NR_{II} family. Numbers indicate the position of the adjacent residue in the respective protein. Va NR_{II}, <u>V. alginolyticus</u> NR_{II}; Kp NR_{II}, <u>K. pneumoniae</u> NR_{II} (McFarlane and Merrick, 1985); Bp NR_{II}, <u>B. parasoniae</u> NR_{II} (Nixon <u>et al</u>., 1986); Ec PhoM, <u>E. coli</u> PhoM (Amemura <u>et al</u>., 1986); Ec CpxA, <u>E. coli</u> CpxA (Albin <u>et al</u>., 1986); Ec EnvZ, <u>E. coli</u> EnvZ (Mizuno <u>et al</u>., 1982); Ec PhoR, <u>E. coli</u> PhoR (Makino <u>et al</u>., 1986); At VirA, <u>A. tumefaciens</u> VirA (Le Roux <u>et al</u>., 1987); Ec CheA, <u>E. coli</u> CheA (Mutoh <u>et al</u>., 1986). Regions that are highly conserved are boxed. (Adapted from McFarlane and Merrick, 1987).

Table 4.5. Family of bacterial two-component regulatory systems that respond to environmental stimuli (Adapted from Ronson *et al.*,1987).

Environmental stimulus	Sensor	Regulator
nitrogen limitation	*glnL*	*glnG*
phosphate limitation	*phoM*	*phoB*
toxic compounds, dyes	*cpxA*	*sfrA*
osmolarity changes	*envZ*	*ompR*
phosphate limitation	*phoR*	*phoB*
plant exudate	*virA*	*virG*
chemical gradients	*cheA*	*cheY*

MacFarlane and Merrick (1987) suggest that it is the N terminal domain that has a sensory function as it is the region which characterizes individual members of a family. Site directed mutagenisis of the K. pneumoniae glnL gene suggests that the C-terminal part of the protein including region three is required for kinase activity while the central part including region two is involved in dephosphorylation (MacFarlane and Merrick, 1987). The Gly rich region is present in region three. This is in keeping with the kinase activity of this region. It is interesting to note that the Gly rich region occurs in all the proteins belonging to the sensor class. The cheA protein has recently been shown to be a kinase of the cheY protein (Wylie et al., 1988). The cheA and cheY genes are required for bacterial chemotaxis. The question remains as to whether or not all the proteins in the sensor class are involved in covalent modification of a regulator protein. Another interesting feature of the cheA protein is that it does not appear to be membrane associated (Stock et al., 1988). In this respect the cheA protein is similar to the NR_{II} protein.

4.3.6 Analysis of the V. alginolyticus glnA-glnL intercistronic region. The nucleotide sequence of the glnA-glnL intercistronic region is given in Fig. 4.13. The glnA gene does not appear to be followed by a Rho-dependent or Rho-independent terminator. This region is approximately 500 bp longer than the corresponding region in E. coli. It would be of interest to investigate the possible involvement

Table 4.6. Aminoacid composition and codon usage for *V. alginolyticus glnA* and *glnL* genes.

Amino acid	codon	*glnA*	*glnL*	Amino acid	codon	*glnA*	*glnL*
F	UUU	7	2	Y	UAU	0	5
	UUC	19	3		UAC	16	1
L	**UUA**	**1**	**9**	H	CAU	0	6
	UUG	**0**	**13**		CAC	13	8
	CUU	**10**	**10**				
	CUC	**0**	**7**	Q	**CAA**	**7**	**26**
	CUA	**16**	**3**		CAG	2	7
	CUG	2	11				
				N	**AAU**	**0**	**8**
I	AUU	3	16		AAC	17	7
	AUC	23	12				
	AUA	**0**	**2**	K	AAA	18	11
					AAG	**7**	**5**
M	AUG	19	8				
				D	GAU	10	11
V	GUU	16	8		GAC	28	9
	GUC	0	6				
	GUA	13	1	E	GAA	27	14
	GUG	5	5		**GAG**	**7**	**7**
S	UCU	16	5	C	UGU	2	2
	UCC	0	0		UGC	1	0
	UCA	**11**	**2**				
	UCG	**3**	**3**	W	UGG	2	2
	AGU	**0**	**6**				
	AGC	2	6	R	CGU	13	8
					CGC	6	7
P	**CCU**	**6**	**2**		**CGA**	**0**	**3**
	CCC	**0**	**3**		**CGG**	**0**	**0**
	CCA	16	8		**AGA**	**0**	**2**
	CCG	2	5		**AGG**	**0**	**0**
T	ACU	6	5	G	GGU	25	5
	ACC	1	5		GGC	9	7
	ACA	**10**	**6**		**GGA**	**0**	**7**
	ACG	**4**	**5**		**GGG**	**0**	**2**
A	GCU	16	4				
	GCC	0	6				
	GCA	22	6				
	GCG	9	11				

Amino acids are designated according to the one letter code. Boldface type correspond to rare codons.

of this region in the regulation of GS expression by oxygen and/or temperature. The presence of a NR_I binding site approximately 30 bp upstream from the presumptive start of the glnL gene suggests that the V. alginolyticus glnL gene is subject to regulation by the NR_I protein. The putative NR_I binding site has 88% homology with the consensus sequence. The glnL genes of E. coli and S. typhimurium are also preceded by a single NR_I binding site (Hanau et al., 1983; Ueno-Nishio et al., 1984). The transcriptional start and the Pribnow sequence of the glnL promoter are overlapped by the NR_I binding site in E. coli and S. typhimurium. Binding of NR_I to this site prevents initiation of transcription at the glnL promoter (Reitzer and Magasanik, 1983). Transcriptional mapping of the V. alginolyticus glnL gene will indicate whether the transcriptional start of the gene is also overlapped by the NR_I binding site.

4.3.7 Codon usage. The amino acid composition and codon usage for V. alginolyticus glnA and glnL genes is listed in Table 4.6. The two genes contain 16 and 37% rare codons respectively. This is in agreement with the observation that regulatory genes usually contain higher percentages of rare codons (24%) than non-regulatory ones (12%) (Konigsberg and Godson, 1983). The percentages of rare codons in the V. alginolyticus genes are however considerably higher than those of the E. coli glnA (6%) and glnL (24%) genes.

CHAPTER FIVE

GENERAL CONCLUSIONS

CHAPTER FIVE

GENERAL CONCLUSIONS

The current study constituted an investigation into the mechanism of regulation and structural organization of the *V. alginolyticus* GS. The cloned *V. alginolyticus glnA* gene was expressed in *E. coli* from a regulatory region contained within the cloned fragment indicating that *E. coli* RNA polymerase was able to recognize *V. alginolyticus* regulatory sequences. An interesting observation was that the cloned *glnA* gene was subject to regulation by oxygen and temperature in addition to nitrogen availability, in *E. coli*. Initial investigations implicated the involvement of a region located downstream of the *glnA* gene in oxygen, temperature and nitrogen regulated expression of the cloned GS. It was subsequently shown that this region was able to regulate the *E. coli* GS *in trans* with respect to nitrogen availability and that the expression of the trans-acting proteins was independent of temperature. DNA sequencing and analysis of the *glnA* region revealed that the *glnL* and *glnG* genes were contiguous with the *glnA* gene. It was also found that the *glnA* promoter region had been truncated during the construction of pRM210 and that only a single Ntr activated promoter was present in this clone. In view of this finding, the regulatory studies carried out on pRM210 in this study need to be reinvestigated with a plasmid containing the entire *V. alginolyticus glnALG* region. The absence of the upstream promoter in pRM210 could explain the

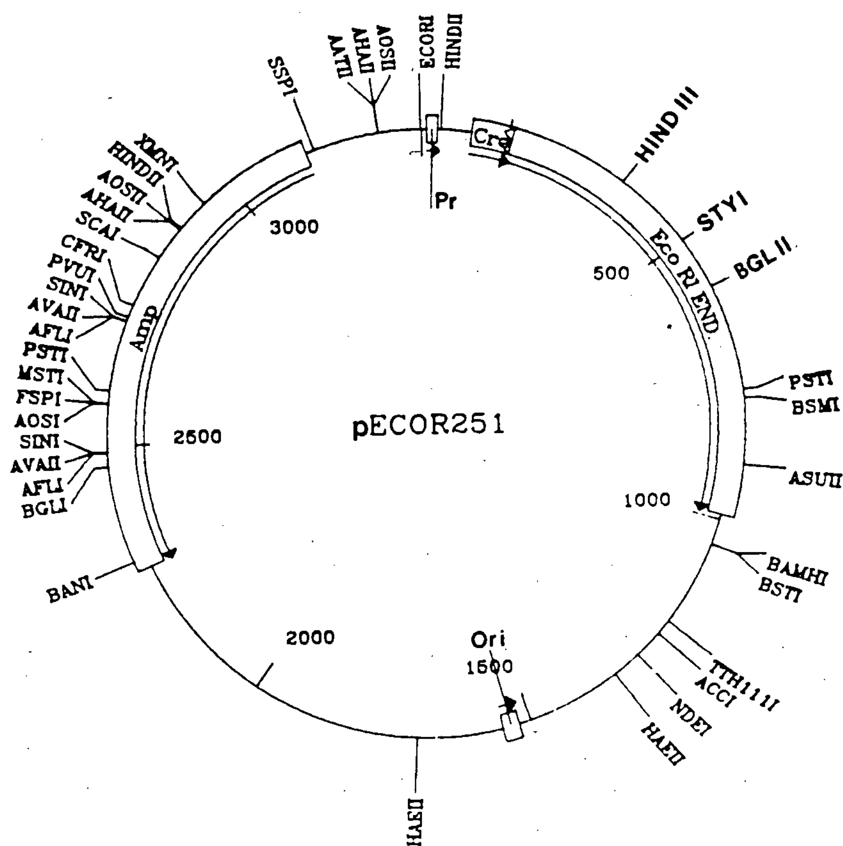
very low basal levels of GS activity produced by pRM211. Plasmid pRM211 is a deletion derivative of pRM210 in which the region encoding the glnL and glnG genes had been deleted. It seems reasonable therefore to conclude that, in pRM210 transcription of GS was initiated at the Ntr regulated promoter and as this promoter has an absolute requirement for the product of the glnG gene, it was inactive in pRM211. The absence of the upstream promoter and the extremely low levels of GS expression by pRM211 did not enable one to establish whether the regulation of expression of GS by oxygen and temperature was independent of the glnL and glnG genes. The occurrence of a region of dyad symmetry with the potential to form secondary stem-loop structures in the promoter region of the glnA gene lends support to the hypothesis that the V. alginolyticus glnA promoter is itself sensitive to changes in oxygen concentration and environmental temperature. Construction of a recombinant containing the entire V. alginolyticus glnALG operon including the complete glnA promoter region will facilitate analysis of the mechanism of regulation of GS in this bacterium.

The structural organization of the V. alginolyticus glnA region parallels that of the enteric bacteria with components that are apparently involved in the cascade regulation of GS. The V. alginolyticus glnA and glnL genes share considerable homology with the E. coli glnA and glnL genes with the exception that V. alginolyticus utilizes a higher percentage of rare codons. In contrast to the glnA

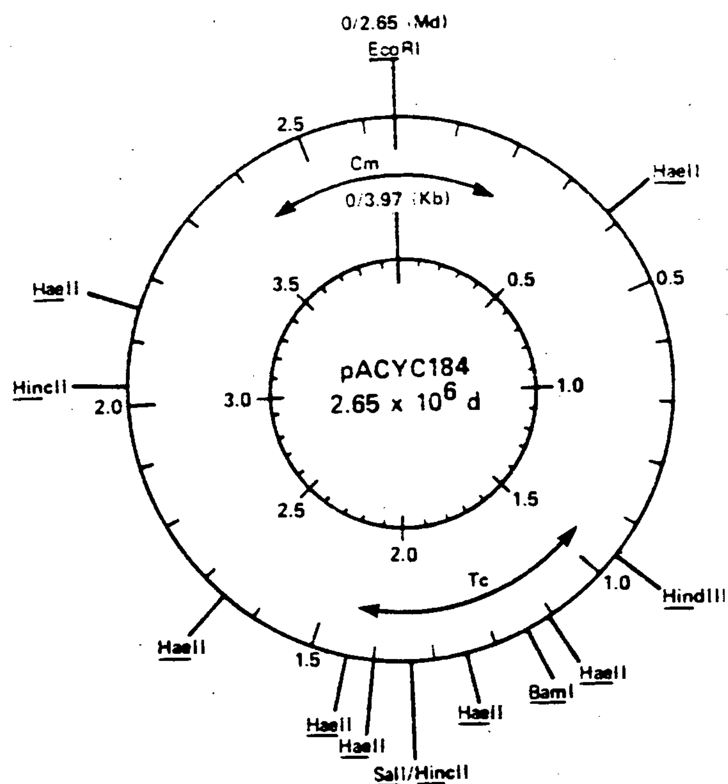
gene, the protease A gene from *V. alginolyticus* showed stronger overall homology to the *Bacillus licheniformis* protease than to the Gram-negative *S. marcescens* (S. Deane, personal communication). These findings are consistent with the observations that certain aspects of nitrogen regulation in *V. alginolyticus* parallel *B. subtilis* rather than the more closely related Gram-negative bacteria (Reid *et al*., 1978; Long *et al*., 1981). The *V. alginolyticus* sucrase gene has also been sequenced (R. Scholle, personal communication) and shares a high degree of homology with the *B. subtilis* sucrase.

APPENDIX A

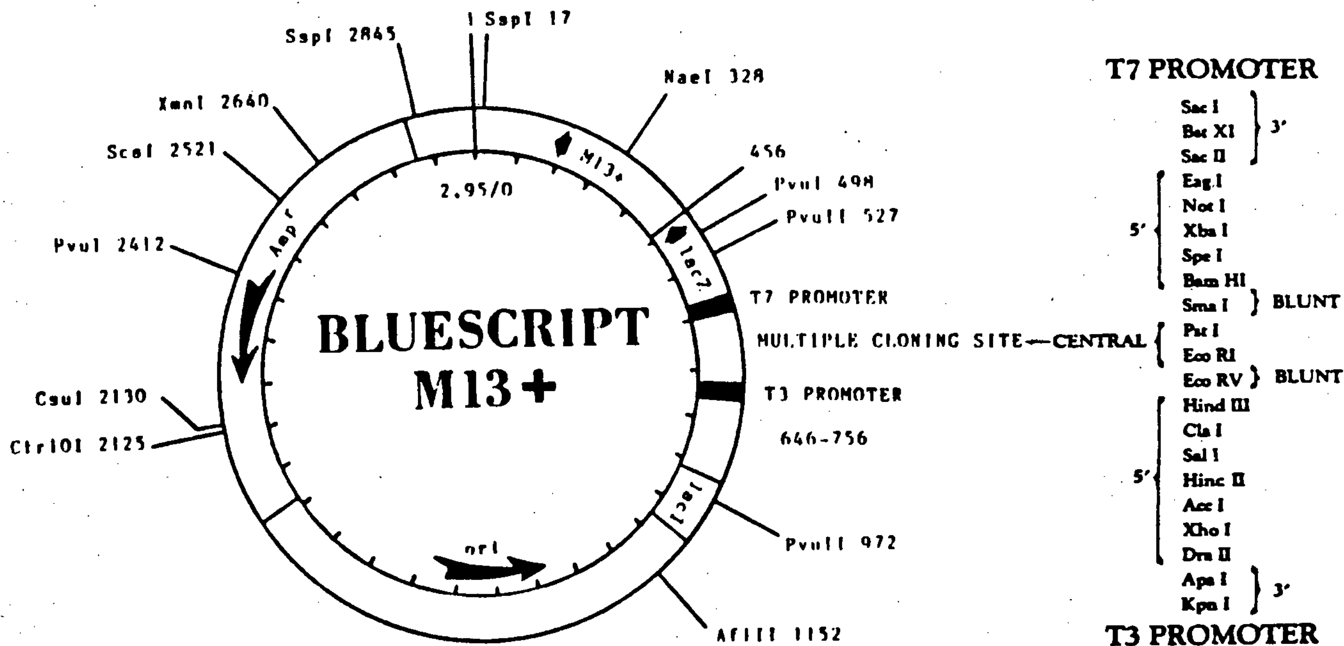
RESTRICTION MAPS OF CLONING VECTORS



Restriction map of pEcoR251 (M Zabeau, Plant GEnetic Systems, Belgium).

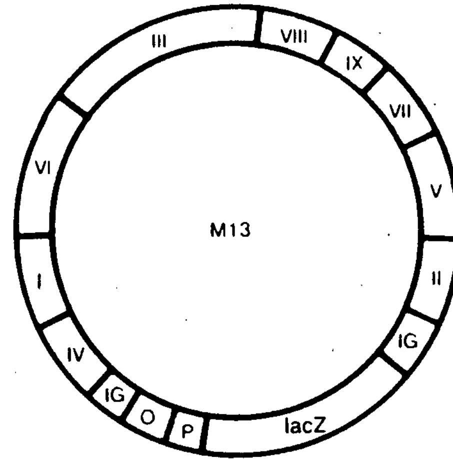


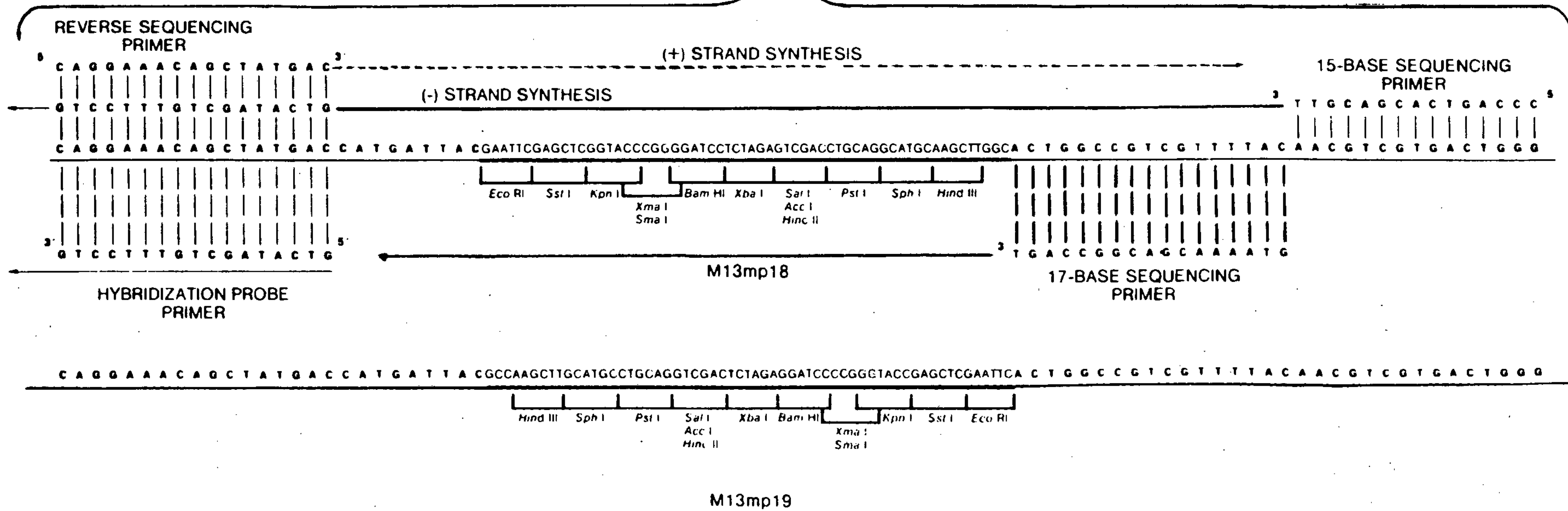
Restriction map of pACYC184 (Chang and Cohen, 1978).



Restriction map of Bluescript (KS) (Stratagene Cloning Systems, San Diego, USA.

Restriction map of pUC18 (Messing and Vierira, 1982).





Restriction maps of M13-mp18 and M13-mp19 (Messing and Vieira, 1982).

APPENDIX B

MEDIA, BUFFERS AND SOLUTIONS

APPENDIX B

MEDIA, BUFFERS AND SOLUTIONS

Acrylamide-bis acrylamide (30:1) stock solution (A-bis-A)

Acrylamide	300 g
N-bis-methylene acrylamide	10 g
Distilled water	to 1000 ml

The solution was stirred with charcoal for 8 h and filtered through Whatman paper No 541.

Ampicillin stock solution

Stock solutions of 25 mg/ml were prepared in distilled water, sterilized by filtration and stored in aliquots at -20^{0}C.

Chloramphenicol stock solution

A stock solution was prepared in 100% ethanol at a concentration of 34 mg/ml. The solution was sterilized by filtration and stored at -20^{0}C for up to one year.

Denhardt's solution (10X)

Ficoll	1% (w/v)
Polyvinylpyrrolidone-40	1% (w/v)
BSA	1% (w/v)

The solution was filter sterilized and stored at -20^{0}C

DNA sample loading buffer

Bromophenol blue	0.25% (w/v)
Glycerol	50% (v/v)
EDTA	100 mM

Denatured salmon sperm DNA

Salmon sperm DNA (Boehringer, Mannheim, West Germany) was resuspended in distilled water at a concentration of 10 mg/ml. The DNA was sheared by passing it several times through an 18-gauge hypodermic needle. The sheared DNA was boiled for 10 min, chilled immediately on ice and stored frozen at -20^{0}C in small aliquots. The DNA was boiled for 5 min and chilled on ice prior to use.

$FeCl_3$ stop reagent

$FeCl_3$	55 g
TCA	20 g
HCL	21 ml
Distilled water	to 1 l

Glutamine stock solution
A stock solution was prepared daily in 20% NaOH at a final concentration of 0.15 mM. The solution was sterilized by filtration.

H agar

Bacto tryptone	10 g
NaCl	8 g
Bacto agar	12 g
Distilled water	to 1 l

H top agar

Bacto tryptone	10 g
NaCl	8 g
Bacto agar	8 g
Distilled water	to 1 l

Lowry reagent A

$CuSO_4$	1% (w/v)

Stable at room temperature

Lowry reagent B

Sodium potassium tartrate	2% (w/v)

Stable at room temperature

Lowry reagent C

Sodium carbonate	2.0 g
NaOH	0.4 g
Distilled water	to 100.0 ml

Prepared fresh before use

Lowry assay reagent
The assay reagent was made up of 1 ml of A + 1 ml of B + 100 ml of C prior to use.

Luria agar (LA)

Bacto tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Bacto agar	15 g
Distilled water	to 1 l

Luria medium

Bacto tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Distilled water	to 1 l

M9 salts (5X)

K_2HPO_4	52.5 g
KH_2PO_4	22.5 g
$(NH_4)_2SO_4$	5.0 g
Sodium citrate	2.5 g
Distilled water	to 1.0 l

The solution was stored under chloroform at room temperature.

Minimal medium

a) M9 salts (5X)	200 ml
Distilled water	200 ml
b) Minimal agar (Oxoid)	15 g
Distilled water	600 ml

The salt solution and agar were autoclaved separately and allowed to cool before mixing. The following supplements were then added:

1 M $MgSO_4$	1.0 ml
1 M Thiamine	0.5 ml
20% (w/v) glucose	10.0 ml

NaCl saturated isopropanol

Equal vol of isopropanol and 5 M NaCl containing 10 mM Tris and 1 mM EDTA, pH 8.5 were mixed. The precipitated NaCl was allowed to settle out of solution and the upper solvent phase was used.

Peptone medium

Tris-HCl buffer (pH 7.6)	100.0 mM
NaCl	2.4%
Bacto peptone	2.5% (w/v)

Resolving gel (10% (w/v)

A-bis-A)	7.5 ml
Running gel buffer	5.6 ml
Distilled water	7.1 ml
Ammonium persulphate (10% (w/v))	50 μl
Temed	25 μl

The ammonium persulphate solution was prepared fresh weekly

Restriction endonuclease buffers (10X)

Low salt buffer:	Tris-HCl (pH 7.4)	100 mM
	$MgCl_2$	100 mM
	DTT	10 mM

Medium salt buffer:	Tris-HCl (pH 7.4)	100 mM
	$MgCl_2$	100 mM
	NaCl	500 mM
	DTT	10 mM

High salt buffer:	Tris-HCl (pH 7.4)	500 mM
	$MgCl_2$	100 mM
	NaCl	1000 mM
	DTT	10 mM

KpnI buffer:	Tris-HCl (pH 7.8)	250 mM
	$MgCl_2$	100 mM
	DTT	10 mM
	BSA	1000 mM

SmaI buffer:	Tris-HCl (pH 8.0)	100 mM
	$MgCl_2$	100 mM
	KCl	200 mM
	DTT	10 mM

RNase free glassware, and solutions
All glassware was treated for 15 h with 0.1% (v/v) diethylpyrocarbonate (DPC) and autoclaved before use. New glassware was used where possible and gloves were worn at all times. All chemicals used were reserved only for experiments with RNA. Where possible solutions were treated overnight with 1% (v/v) DPC. Treated solutions were autoclaved before use. Solutions containing Tris were not treated with DPC, as it is highly unstable in the presence of Tris.

SB buffer

Imidazole-HCl (pH 7.5)	20 mM
$MgCl_2$	10 mM
β-mercaptoethanol	2 mM

Stacking gel

A-BIS-A	1.00 ml
Stacking gel buffer	1.25 ml
Distilled water	7.50 ml
Ammonium persulphate (10% (w/v)	50.00 μl
Temed	25.00 μl

SSC (20X)

NaCl	3.0 M
Sodium citrate	0.3 M

Sucrose solution for gradient centrifugation

Sucrose	25% (w/v)
NaCl	1000 M
Tris-HCl (pH 8.0)	20 mM
EDTA	5 mM

Tetracycline

A stock solution of 12.5 mg/ml was prepared in ethanol/water 50% (v/v) and stored in aliquots at -20^{0}C in the dark.

TE buffer (pH 7.6)

Tris-HCl (pH 7.6)	10 mM
EDTA (pH 8.0)	1 mM

TE-equilibrated phenol

An unopened bottle of phenol (Merck, Darmstadt, West Germany) was melted at 65^{0}C. 8-Hydroxyquinoline was added to a final concentration of 0.1% (w/v), and the phenol equilibrated against TE. The equilibrated phenol was stored at 4^{0}C in the dark.

Tris acetate buffer (50X)

Tris-HCl	242.0 g
Glacial acetic acid	57.1 ml
EDTA	37.2 g

TY broth

Bacto tryptone	16 g
Yeast extract	10 g
NaCl	5 g
Distilled water	to 1 l

APPENDIX C

GENERAL DNA TECHNIQUES

APPENDIX C

GENERAL DNA TECHNIQUES

C.1 EXTRACTION OF *E. coli* PLASMID DNA
- C.1.1 Small scale ("miniprep") extraction
- C.1.2 Large scale ("maxiprep") extraction

C.2 EXTRACTION OF PHAGE DNA
- C.2.1 Preparation of single-stranded template
- C.2.2 Preparation of M13 RF DNA

C.3 PURIFICATION OF DNA

C.4 RESTRICTION ENZYME DIGESTION

C.5 DNA GEL ELECTROPHORESIS

C.6 DNA FRACTIONATION THROUGH SUCROSE DENSITY GRADIENTS

C.7 ELUTION OF DNA FROM AGAROSE GELS

C.8 LIGATION OF DNA

C.9 PREPARATION OF COMPETENT *E. coli* CELLS
- C.9.1 Routine method for *E. coli* strains
- C.9.2 Preparation of competent *E. coli* LK111 cells

C.10 TRANSFORMATION OF COMPETENT *E. coli* CELLS
- C.10.1 Transformation by plasmid DNA
- C.10.2 Transformation by M13 RF DNA

C.11 RADIOACTIVE LABELLING OF DNA PROBES

C.12 IMMOBILIZATION OF DNA ON NITROCELLULOSE

C.13 HYBRIDIZATION OF DNA

C.14 UNIDIRECTIONAL DIGESTION OF DNA WITH EXONUCLEASE III

C.1 EXTRACTION OF *E. coli* PLASMID DNA

C.1.1 Small scale ("miniprep") extraction. *E. coli* strains containing plasmids were grown in LB (Appendix B) containing the relevant antibiotic (Appendix B) to maintain selective pressure. Plasmid DNA was isolated from 1.0 ml of a saturated culture employing the method of Ish-Horowicz and Burke (1981). Bacterial cells were harvested by centrifugation (20 sec) in an Eppendorf microfuge, resuspended in 100 μl of Solution I (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0) and maintained at room temperature for 5 min. 200 μl of Solution II (0.2 N NaOH, 1% (w/v) SDS) was then added, the mixture mixed gently and placed on ice for 5 min. 150 μl of precooled Solution III (5 M potassium acetate, pH 4.8) was added, mixed gently and maintained at 4^0C for 5 min. Precipitated protein, SDS and chromosomal DNA was removed by centrifugation (5 min) in an Eppendorf microfuge. Two volumes of ethanol were added to the supernatant, the mixture held at room temperature for 2 min and the nucleic acids pelleted by centrifugation (3 min) in an Eppendorf microfuge. The pellet was resuspended in 100 μl TE buffer (Appendix C), extracted with an equal volume of phenol (Appendix A) and precipitated with ethanol. The resulting pellet was dried in a vacuum centrifuge and resuspended in 100 μl TE buffer. Plasmid DNA prepared according to this protocol proved to be sufficiently pure for restriction endonuclease digestion.

C.1.2 Large scale ("maxiprep") extraction. For large scale plasmid extraction, 200 ml of an overnight culture in LB was

harvested. The procedure employed was that of Ish-Horowicz and Burke (1981) scaled up (20-fold) for larger volumes. The nucleic acid pellet obtained after ethanol precipitation was resuspended in 4.5 ml TE (Appendix B) and subjected to CsCl-EtBr equilibrium density gradient centrifugation (Appendix C.3)

C.2 EXTRACTION OF PHAGE DNA

C.2.1 Preparation of single-stranded template. Single-stranded M13 DNA was prepared according to the procedure described in the Amersham (Amersham International plc, UK) M13 cloning and sequencing handbook. A fresh overnight culture (1 ml) of host cells (E. coli JM105) in 2X YT medium (Appendix B) was diluted into prewarmed (37^0C) 2X YT medium (100 ml) and aliquots (1.5 ml) dispensed into sterile culture tubes. Sterile wooden cocktail sticks were used to inoculate the growth medium with infected cells from individual plaques. Cultures were grown at 37^0C for 6 h with vigorous shaking. The supernatant was recovered by centrifugation (10 min) in an Eppendorf microfuge. The pellet of infected E. coli JM105 cells was retained for extraction of M13 RF DNA. Residual bacterial cells in the supernatant were pelleted by centrifugation as before. The supernatant was incubated with 200 μl of NaCl (2.5 M) containing polyethylene glycol 6000 (20%) (w/v) at 22^0C for 15 min. The viral pellet was recovered by centrifugation (10 min) in an Eppendorf microfuge, taking care to remove all remaining traces of PEG, and resuspended in 100 μl TE (Appendix B). Phenol (50 μl) was added and the mixture

allowed to stand at 22^0C for 15 min. The aqueous phase was recovered by centrifugation, the DNA ethanol precipitated, washed (70% (v/v) ethanol) and the pellet resuspended in 50 μl TE. Samples were stored frozen (-20^0C) until required.

C.2.2 Preparation of M13 RF DNA. Small and large scale RF DNA extractions were as described by Ish-Horowicz and Burke (1981). Small scale RF DNA preparation involved the extraction of DNA from bacterial pellets obtained in the preparation of single-stranded template (Appendix C.2.2). For large scale RF DNA extraction 2X YT medium (200 ml) was inoculated with phage supernatant and a fresh culture (2 ml) of E. coli JM105 cells grown in 2X YT medium. The culture was grown at 37^0C for 12-15 h with shaking and RF DNA extracted from the bacterial pellet.

C.3 PURIFICATION OF DNA

DNA preparations were purified by centrifugation in CsCl-EtBr gradients. Ethidium bromide and cesium chloride were added to final concentrations of 250 μg/ml and 1 g/ml respectively, the solution cleared by centrifugation (15000 rpm/15 min) and the refractive index of the supernatant adjusted to 1.394. The extract (5 ml) was sealed in a Beckman Quickseal ultracentrifuge tube and centrifuged (55000 rpm/8 h) at 15^0C in a Beckman VTi rotor. The plasmid band was visualised with long wave ultraviolet (UV) light (350 nm) and the fluorescent band recovered. The Et-Br was extracted with NaCl-saturated isopropanol (Appendix B). Two volumes of water were then added and the nucleic acids

precipitated with an equal volume of isopropanol. The DNA was pelleted by centrifugation (10 min) in an Eppendorf microfuge. The nucleic acid concentration was estimated spectrophotometrically. One absorbance unit (260 nm) was considered equivalent to 50 μg DNA/ml (Davis *et al*., 1980)

C.4 RESTRICTION ENZYME DIGESTION

Established techniques for the analysis of DNA (Maniatis *et al*., 1982) were followed. The commercial suppliers of restriction endonucleases used are listed in Appendix G. Restriction enzyme digestions of DNA were carried out using low, medium or high salt restriction buffers (Appendix B) according to the salt preference of the enzyme as specified by the suppliers. *Sma*I and *Kpn*I were exceptions to the rule requiring *Sma*I and *Kpn*I specific buffers (Appendix B) respectively. The incubation temperatures for restriction digests were as specified by the commercial suppliers. Double and triple digestions were performed simultaneously if the salt and temperature requirements of the enzymes were identical. Where the conditions were dissimilar, restrictions were carried out sequentially using the enzyme with the lowest salt optimum and the highest temperature tolerance first. The incubation mixture and temperature were adjusted before addition of the second (and third) enzyme. Routinely, plasmid DNA digests were incubated for 2 h and chromosomal DNA digests for 4 h. The digestion time for *Bgl*II however, was kept to a minimum to reduce phosphatase activity of *Bgl*II. Optimum conditions for *Bgl*II and partial *Sau*3A restrictions were determined from pilot

digests relating the extent of digestions as a function of time. Restriction digests consisted of 2 μl (10X) restriction buffer, 1 μg of DNA and 2 units of restriction endonuclease in a final volume of 20 μl. For bulk digests, all components were scaled up accordingly. Enzyme activity was terminated by the addition of 1/6 vol of EDTA containing DNA sample loading buffer (Appendix B). Restricted DNA not intended for electrophoretic analysis was phenol extracted, isopropanol precipitated and resuspended in TE buffer before being subjected to further manipulation.

C.5 DNA GEL ELECTROPHORESIS

Electrophoresis was carried out using a horizontal gel system with Tris-Acetate (TAE) buffer (Appendix B). The concentration of agarose (Sigma type II) varied from 0.6% (w/v) for the analysis of large DNA fragments to 2.0% (w/v) for the detection and sizing of smaller DNA fragments (Maniatis et al., 1982). An agarose concentration of 0.8% (w/v) was used for routine analysis of DNA. Electrophoresis was carried out toward the anode at 10 V/cm for rapid analysis in minigels or 2.7 V/cm overnight through large gels. Molecular weight markers were restriction digests of lambda DNA. Gels were stained with ethidium bromide (0.5 μg/ml buffer) and the DNA visualised using a 254 nm wavelength Transilluminator (Chromato-Vue Model TS-15, UV Products Inc., San Gabriel, CA, USA). The gels were photographed using a Polaroid CU-5 Land camera and Polaroid Land Pack 667 film. Exposures were of 1-5 sec duration at an aperture of f-4.5. Molecular weights were determined by

measuring the mobilities of individual bands relative to the lambda markers.

C.6 DNA FRACTIONATION THROUGH SUCROSE DENSITY GRADIENTS

The standard method of Maniatis et al. (1978) was followed. Gradients (38 ml) were prepared in Beckman SW2 ultracentrifuge tubes. Sucrose (10-40% (w/v) gradients were established after two cycles of freezing (-20^{0}C) and thawing (4^{0}C) of a 25% (w/v) sucrose solution (Appendix B). Samples (100 μg) were centrifuged at 20000 rpm for 20 h at 15^{0}C in a Beckman SW 27 rotor. Fractions (0.9 ml) were eluted from the bottom of the tube. Sodium perchlorate (5 M) was added to a final concentration of 500 mM and the DNA precipitated with 1/2 vol isopropanol. The DNA pellet was resuspended in TE buffer (100 μl) and aliquots (2 μl) analyzed by agarose gel electrophoresis.

C.7 ELUTION OF DNA FROM AGAROSE GELS

The method of Dretzen et al. (1981) was modified for use of DEAE membrane (NA45, 0.45 μm membrane, Schleicher and Schuell, Dassel, Germany). DNA fragments of interest were resolved by electrophoresis and visualized by EtBr fluorescence using long wave (350 nm) UV light. Horizontal slits were made directly below the band to be recovered and a piece of DEAE membrane of the appropriate size was inserted into the slit. Contamination by slower migrating fragments was prevented by the insertion of a piece of membrane or excision of the gel above the fragment to be eluted. Electrophoresis was continued at 10 V/cm until the

DNA had entered the membrane. Transfer of the DNA was verified by observation on a UV Transilluminator. Elution of membrane bound DNA was achieved in a high salt buffer (20 mM Tris-HCl, 1mM EDTA, 1.5 M NaCl, pH 7.5) at 37^{0}C in 2-15 h. This was verified by observation of the DEAE membrane under UV illumination. DNA in solution was recovered by ethanol precipitation and resuspended in TE buffer.

C.8 LIGATION OF DNA

A 1:1 molar ratio of vector:insert DNA molecules was found to be optimal for the construction of a V. alginolyticus genomic library. For subcloning experiments and cloning of single fragments, a variety of concentrations and ratios of vector DNA to insert DNA was employed depending on the size of the fragment to be cloned and the number of possible ligation products. Typically, ligation mixes contained 100-200 ng DNA, 1 μl ATP (10 mM) (Appendix B), 1 μl 10X ligation buffer (0.5 mM Tris·HCl pH 7.4, 0.1 M $MgCl_2$, 0.1 M dithiothreitol) and 0.5-1.0 units of T4 DNA ligase in a final volume of 10 μl. Reaction mixes were incubated at room temperature for 2 h or at 15^{0}C overnight for sticky and blunt end ligations respectively.

C.9 PREPARATION OF COMPETENT E. coli CELLS

C.9.1 General method. Competent cells were prepared by the method of Cohen et al. (1972) with modifications as described by Dagert and Ehrlich (1979). A 1/100 dilution of an overnight culture of E. coli cells in LB was made into fresh LB and the culture incubated at 37^{0}C, with aeration,

until early exponential phase of growth was reached (A_{600nm}=0.2). The bacterial culture was chilled rapidly and all consequent preparative procedures were performed at 4^0C. The cells were harvested by centrifugation (5000 rpm/10 min), resuspended in 1/2 the growth volume of cold 100 mM $CaCl_2$, 15% (v/v) glycerol solution and allowed to age for 2-15 h depending on the particular *E. coli* strain preference. Competent cells were stored at -70^0C and thawed at 4^0C before use.

C.9.2 Preparation of competent *E. coli* LK111 cells. The method of Zabeau and Stanley (1982) was followed. A fresh overnight culture in LB of *E. coli* LK111 cells was diluted 1/1000 in fresh LB. The culture was grown shaking at 37^0C until a cell density of A_{600nm}=0.2 was reached. The cells were harvested by centrifugation (8000 rpm/10 min), resuspended in an equal volume of cold 100 mM $MgCl_2$ and pelleted by centrifugation (8000 rpm/10 min). The pellet was resuspended in 1/2 the growth volume of cold 100 mM $CaCl_2$ and maintained at 4^0C for 20 min. The cells were then harvested and resuspended in 1/10 the growth volume of cold 100 mM $CaCl_2$. *E. coli* LK111 cells were stored at 4^0C and remained competent for up to 3 days.

C.10 TRANSFORMATION OF COMPETENT *E. coli* CELLS

C.10.1 Transformation by plasmid DNA. Competent *E. coli* cells (100 μl) were transformed with 2-10 μl of ligation mixture containing 20-100 ng of DNA. The transformation mixture was maintained at 4^0C for 10 min, heated to 42^0C

for 2 min to induce uptake of DNA by competent cells and once again chilled at 4^0C. To each sample, 1 ml of LB was added and the transformed DNA allowed to express at 37^0C with shaking for 1 h. Typically, 50-100 μl of expression mix was plated onto LA plates containing an appropriate antibiotic (Appendix B) and incubated aerobically at 37^0C overnight. The expression mix was stored at 4^0C and plated at a higher or lower cell density where necessary. Putative recombinants were analyzed by small scale plasmid extraction (Ish-Horowicz and Burke, 1981)and restriction analysis.

C.10.2 Transformation by M13 RF DNA. Transformation of competent E. coli cells by M13 RF DNA was as described by Cohen et al. (1972) and Hanahan (1983). Competent E. coli JM105 cells (100 μl) were transformed with 5-10 μl ligation mixture containing 50-200 ng of DNA. The transformation mixture was maintained at 4^0 C for 40 min, heatshocked at 42^0C for 3 min and returned to 4^0C. The heatshocked cells and DNA were then mixed with IPTG (40 mM), Xgal (0.8%) (w/v), host cells (300 μl) grown to an A_{600nm} of 0.2 and H top agar (3 ml) (Appendix B) and incubated aerobically at 37^0C for 12-15 h. An antibiotic amplification period was not required for RF DNA. Phage particles harbouring inserts gave colourless plaques on Xgal plates, resulting from insertional inactivation of the β-galactosidase gene.

C.11 RADIOACTIVE LABELLING OF DNA PROBES

DNA probes were labelled to high specific activity by nick translation (Rigby _et al_., 1977). The reagents were obtained from Amersham (Amersham Int., UK, BB.5025) in a kit form and the reaction was carried out according to the supplier's instructions. The nick translation reaction mixture was incubated at 15^0C for 2 h and the labelled DNA recovered in the void volume of a Sephadex G-50 spin column (Maniatis _et al_., 1982). Specific activities of labelled probes were determined by Cherenkov counting on a Packard scintillation counter. Probes were either used immediately or stored in lead canisters at 4^0C until needed. Denaturation of labelled DNA was achieved by boiling in a fume hood for 10 min.

C.12 IMMOBILIZATION OF DNA ON NITROCELLULOSE

Transfer of DNA from agarose gels to Gene Screen hybridization transfer membrane (New England Nuclear Corp., Boston, USA) was by the method of Smith and Summers (1980). Following electrophoresis, DNA in the agarose gel was denatured by soaking the gel twice for 15 min in 2 volumes of 0.5 M NaOH containing 1.5 M NaCl. The gel was neutralized by soaking twice for 30 min in 2 vol of 1 M ammonium acetate, 0.02 M NaOH. The gel was then placed on a clean glass surface. Three pieces of Whatman 3 MM filter paper, also cut to the gel dimensions and soaked in 1 M ammonium acetate, 0.02 M NaOH, were layered on the membrane. A 4 cm layer of dry paper towel was placed on the filter paper. A light weight (2 kg) was placed on the layer of

paper towel and transfer allowed to proceed for 2-15 h. The filter was than rinsed in 2X SSC (Appendix B), air dried and baked in vacuo for 2 h.

C.13 HYBRIDIZATION OF DNA

Standard procedures for hybridization of Southern filters (Maniatis et al., 1982) were followed. The baked membrane was soaked in 6X SSC (Appendix B) and transferred to a plastic container or heat sealable plastic bag. Prehybridization was carried out at 60^0C for 2-16 h in an excess volume of prehybridiazation fluid (6X SSC, 0.5% (w/v) SDS, 0.01 M EDTA, 5X Denhardt's solution, 100 µg/ml denatured salmon sperm DNA (Appendix B)). The excess fluid was decanted, the denatured probe ($1x10^7$cpm) added and hybridization continued at 60^0C for 12-16 h. The membrane was washed sequentially at room temperature in 2X SSC containing 0.5% (w/v) SDS for 5 min and 2X SSC containing 0.1% (w/v) SDS for 15 min. The final wash was at at 68^0C for 1-2 h. The wet membrane was exposed under Kodak XAR-5 autographic film in an X-ray cassette containing a Fuji X-ray intensifying screen. Exposure was for 1-3 d at -70^0C. The film was developed using Kodak GBX X-ray developer and fixer according to the manufacturer's specifications.

C.14 UNIDIRECTIONAL DIGESTION OF DNA WITH EXONUCLEASE III

A modification of the method of Henikoff (1987) was employed. The target DNA was cloned into the polylinker region of an appropriate vector such that cleavage at two unique restriction enzyme sites in the cloning cassette

would lead to a 5′-protrusion or blunt end adjacent to the insert and a 3′-protrusion adjacent to the sequencing primer site. Double-stranded DNA (5-10 μg) was cut to completion with the two restriction endonucleases leaving a 5′-protrusion which was susceptible to exonuclease III attack and a 3′-protrusion protecting the vector. The double cut DNA was phenol extracted, ethanol precipitated and resuspended in exonuclease III buffer (66 mM Tris·HCl, pH 8.0, 0.66 mM $MgCl_2$) at a concentration of 10 ng/ul. Exonuclease III digestions were performed at 37^0C and the reaction was commenced with the addition of enzyme (5 units/μl). At 30 sec intervals, 40 μl samples of enzyme digest were removed and diluted 3.5-fold with cold S1 exonuclease mix (40 mM KOAc pH 4.6, 280 mM NaCl, 3 mM $ZnSO_4$, 6% (w/v) glycerol, 2 units/μl S1 exonuclease). After incubation at room temperature for 10 min, 1/10 vol S1 exonuclease S1 stop mix (0.3 M Tris·OH, 0.05 M EDTA) was added. The reaction mixes were maintained at 70^0C for 10 min and samples (50 ng DNA) were examined by agarose gel electrophoresis to determine the extent of digestion. One tenth vol Klenow mix (20 mM Tris·HCl pH7.6, 15 mM $MgCl_2$, 0.04 units/μl Klenow polymerase) was added and the reaction mixes maintained at room temperature for 3 min. To each reaction mix 2/10 vol of a mixture of dNTP's (0.125 mM) was added and the reaction mixes maintained at RT for 5 min. The DNA was ethanol precipitated, resuspended in TE (10 ng/μl) and recircularized in the presence of T4 DNA ligase. Ligation mixes consisted of 100 ng DNA, 1 μl 10X ligation buffer (0.5 M Tris·HCl pH 7.4, 0.1 M $MgCl_2$, 0.1 M

dithiothreitol) and 0.25 units T4 DNA ligase in a final volume of 10 μl. Ligations were carried out at room temperature for 2 h. Competent *E. coli* cells were transformed with 50 ng DNA. Clones were verified by restriction endonuclease analysis and agarose gel electrophoresis.

APPENDIX D

COLORIMETRIC ASSAYS

APPENDIX D

COLORIMETRIC ASSAYS

D.1 PREPARATION OF CTAB PERMEABILIZED CELLS

D.2 PREPARATION OF SONICATED CELL EXTRACTS

D.3 GLUTAMINE SYNTHETASE ASSAY

D.4 HISTIDASE ASSAY

D.5 PROTEIN ESTIMATIONS

All reagents are described in Appendix B unless otherwise stated.

D.1 PREPARATION OF CTAB PERMEABALIZED CELLS

CTAB (0.1 mg/ml) was added to mid exponential phase cultures (100 ml) and the cultures maintained at the growth temperature, with shaking for 5 min. The bacterial cells were harvested by centrifugation (5000 rpm/10 min) and resuspended in 100 ml NH_4Cl (1% (w/v)). The cells were harvested as before, the pellet was resuspended in 1 ml SB buffer and maintained at 4^0C.

D.2 PREPARATION OF SONICATED CELL EXTRACTS

Mid-exponential phase cultures (100 ml) were harvested by centrifugation (5000 rpm/10 min) and the bacterial pellet was resuspended in 1 ml SB buffer. The cells were ruptured by sonicating for 3 X10 sec periods with 30 sec intervals. Cell debris was removed by centrifugation (18000 rpm) at 4^0C for 45 min. The supernatant was recovered and stored on ice.

D.3 GLUTAMINE SYNTHETASE ASSY

A modification of the γGT assay of Shapiro and Stadtman (1970) was employed. The assay system consisted of 1 μmol $MgCl_2$, 0.2 μmol β-mercaptoethanol, 9.2 μmol imidazole-HCl, 9.6 μmol hydroxylamine-HCl, 0.14 μmol $MnCl_2$, 13.8 μmol potassium arsenate, 0.24 μmol NaADP and 10 μmol glutamine in a total volume of 550 μl. Reaction mixes were prepared freshly as follows :

SOLUTIONS ADDED		REACTION TEST	MIXES BLANK
1.00 M Imidazole-HCl pH 7.15	(ml)	1.500	1.500
0.80 M Hydroxylamine-HCl	(ml)	0.246	0.246
0.10 M $MnCl_2$	(ml)	0.030	0.030
0.28 M Potassium arsenate	(ml)	1.000	-
0.04 M NaADP	(ml)	0.1000	-
Distilled water	(ml)	5.220	6.320

Glutaminase catalyzed γ-glutamylhydroxymate production was corrected for by substraction ofγGT produced in the blank incubation mixes lacking in ADP and arsenate. The pH of the reaction mixes was adjusted to 7.4. Incubation mixtures consisted of 400 μl of test and blank reaction mixes respectively and 100 μl of suitably diluted CTAB permeabalized cells or sonicated cell extracts. The incubation mixtures were prewarmed at 37^0C for 5 min and the reaction was started with the addition of 50 μl of 0.2M glutamine. Incubations were carried out at 37^0C for 15 min and the reaction was terminated by the addition of 1 ml of $FeCl_2$ stop reagent. The mixtures were then cleared by centrifugation (1 min) in an Eppendorf microfuge and the absorbance values of the coloured iron-hydroxyamate complex recorded at 540 nm on a Beckman DU-40 spectrophotometer. A γGT standard curve was established in the range 0-1.2 μmol γGT/100 μl.

D.4 HISTIDASE ASSAY

A modification of the methods of Hartwell and Magasanik (1963) and Chasin and Magasanik (1968) was eployed. The assay system consisted of 20 μmol potassium phosphate buffer, 100 μmol diethanolamine-HCl and 20 μmol L-histidine in a final volume of 1 ml. Incubation mixtures were prepared as follows:

SOLUTIONS ADDED	TEST INCUBATIONS of varying enzyme dilutions	BLANK
0.05 M Potassium phosphate buffer pH 7.4 (ml)	0.4	0.4
1.00 M Diethanolamine-HCl (ml)	0.1	0.1
0.10 M L-Histidine (ml)	0.2	-
Distilled water (ml)	0.2	0.4
Toluene (25%)(v/v) permeabalized cells (ml)	0.1	0.1

The reaction was started with the addition of L-hisidine and incubations were carried out at 37^{0}C for 15 min. Histidase activity was terminated with 1 ml of saturated sodium tetrahydroborate and the absorbance values recorded at 277 nm on a Beckman DU-40 spectrophotometer. The production of urocanic acid was estimated by reference to a urocanic acid standard curve prepared in the range 0-90 μmol/100 μl.

D.5 PROTEIN ESTIMATIONS

Suitable dilutions of samples were prepared in 0.1 ml aliquots and the total protein content was determined by the method of Lowry et al.(1951). To each 0.1 ml aliquot, 1 ml of Lowry assay reagent was added and the mixture allowed to stand for 10 min. 0.5 ml of Folin and Ciocalteu reagent (50%)(v/v) was added, the solutions mixed and left for 30 min for colour development. The absorbance values were then recorded at 600 nm, on a Beckman DU-40 spectrophotometer, against a reagent blank. A callibration curve was constructed by preparing various dilutions of bovine serum albumin (BSA) in the range 0-200 μg protein/ml.

APPENDIX E

IMMUNOCHEMISTRY

E.1 WESTERN BLOTTING
E.1.1 Sodium dodecyl sulphate/polyacrylamide gel electrophoresis
E.1.2 Electrophoretic transfer of proteins to Gene-Screen
E.1.3 Detection of antigens with enzyme-labelled antibodies

E.2 ENZYME-LINKED IMMUNOSORBENT ASSAY

E.1 WESTERN BLOTTING

E.1.1 Sodium dodecyl sulphate/polyacrylamide gel ectrophoresis. The method of Laemmli (1970) was employed. The ratio of acrylamide to methylene bisacrylamide was 36.5:1.0 (w/w). Polymerization was initiated by the addition of 10% (w/v) ammonium persulphate. A 10% (w/v) resolving gel (Appendix B) was cast in a Hoeffer gel apparatus SE600 with 1.5 mm spaces and the monolayer was carefully overlaid with distilled water. A 5% (w/v) stacking gel (Appendix B) was then cast and a comb presoaked in ammonium persulphate inserted into the monolayer.

Protein extracts were mixed with an equal volume of sample buffer (4 μl 0.125 M HCl, 2.5 μl 10% (w/v) SDS, 2 μl glycerol, 1.5 μl distilled water) and the mixture was heated at 100°C for 5 min. Elecrophoresis was carried out toward the anode with 1.5 M Tris-HCl buffer pH 8.8 containing 0.4% (w/v) SDS as tank buffer. The samples were layered under the electrophoresis buffer. Bromophenol blue was used as the tracker dye. Molecular weight markers in the range 14000-94000 kD were obtained from Pharmacia Fine Chemicals, Uppsula, Sweden. Electrophoresis was carried out at 100 V until the sample entered the resolving gel, the current was then adjusted to 10 mA per gel with constant voltage and electrophoresis was terminated when the dye reached the bottom of the gel. Gels were run in duplicate and the resolved proteins either electroblotted or visualized by staining. The gels were stained for 1 h in 0.05% (v/v) acetic acid containing 25% (v/v) propanol. Destaining was

by diffusion in 7.5% (v/v) acetic acid containing 25% (v/v) propanol with several changes of destain.

E.1.2 Electrophoretic transfer of proteins to Gene Screen. The Electroblotting procedure followed was that of Rybicki and von Wechmar (1982). A piece of Gene Screen membrane, the size of the gel, was soaked in transfer buffer (20 mM Tris base, 150 mM glycine and 20% (v/v) methanol) for 20 min and placed on the gel. The gel was then sandwiched between two pieces of filter paper (Whatman 3 MM), presoaked in transfer buffer, followed by 2 Scotch-Brite pads soaked in transfer buffer. The "sandwich" of filter papers, gel and Gene Screen membrane was then placed in a Hoeffer TE transfer apparatus with the Gene Screen membrane positioned between the gel and the anode. Electrophoresis was carried out toward the anode with transfer buffer. Transfer of proteins was continued at 0.1 A for 12-16 h at 4^{0}C.

E.1.3 Detection of antigens with enzyme-labelled antibodies. Following electrophoretic transfer of resolved proteins, the Gene Screen membrane was soaked in an excess volume of blocking solution (2% (w/v) BSA, 0.05% (v/v) Tween 20, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4) for 90 min at 37^{0}C with gentle shaking. The filter was then incubated with a minimum volume of affinity purified antibody for 90 min at 37^{0}C with constant agitation. This was followed by three washes in 0.15 M NaCl containing 0.05% (v/v) Tween 20. The washes were of 5 min duration at 22^{0}C with constant shaking. The membrane was then incubated with a 10^{-3} dilution of goat

anti-rabbitIg-horseradish peroxidase conjugate (Seravac, Cape Town, South Africa) containing 0.9% (w/v) NaCl, 0.05% (w/v) BSA and 10 mM Tris-HCl (pH 7.4). Incubation was continued for 90 min at 22^0C and the filter subjected to three washes in 0.15 M NaCl containing 0.05% (v/v) Tween 20 as before. The antigens were detected colorimetrically following incubation in substrate buffer (5% (v/v) H_2O_2, 200 mM NaCl, 50 mM Trsi-HCl, pH 7.4) for 5-30 min at 22^0C. Termination of the enzyme reaction was achieved by washing the membrane in distilled water.

E.2 ENZYME-LINKED IMMUNOSORBENT ASSAY

ELISAs (Clark and Adams, 1977) were carried out on sonicated E. coli cell extracts (Appendix D), of known protein concentration (Appendix D). Reaction volumes of 200 µl were used for all steps except the substrate reaction, when 300 µl volumes were used. Microtitre plates (Dynatech, Plochingen, FRG) were coated with serial dilutions of E. coli cell extracts for 90 min at 37^0C. Plates were then washed three times with PBS-T (0.01 M phosphate buffer, 0.9% (w/v) NaCl, pH 7.4 containing 0.05% (v/v) Tween-20) and incubated with PBS-T containing 0.2% (w/v) BSA for 90 min at 37^0C. Plates were washed as before and GS antibodies allowed to react with the trapped antigen for 90 min at 37^0C. After washing with PBS-T, horseradish peroxidase-labelled goat anti-rabbit IgG (GAR) antiserum (Seravac, Cape Town, South Africa) was added to each well and incubated for 90 min at 37^0C. After further washing, the bound enzyme conjugate was detected colorimetrically by adding substrate

(1 mg/ml p-nitrophenyl phosphate in 10% (v/v) diethanolamine, pH 9.8). The reaction was followed photometrically in a Titertek Multiskan plate reader (Flow Laboratories).

APPENDIX F

AMINO ACID SYMBOLS

Amino acid	3-letter code	1-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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