REGULATION OF *CLOSTRIDIUM ACETOBUTYLICUM* GLUTAMINE SYNTHETASE *glnA* GENE

by

Ivo Fierro-Monti
Department of Microbiology
University of Cape Town

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CERTIFICATION OF THE SUPERVISORS

In terms of paragraph GP 8 of "General Rules for the degree of Doctor of Philoshophy (PhD)" I, as a supervisor of the candidate Ivo Fierro-Monti, certify that I approve of the incorporation in this thesis of material that has already been published or submitted for publication.

Professor D.R. Woods
Deputy Vice Chancellor (Research)
Director of
Microbiology Research Units
University of Cape Town

Senior Lecturer Dr. S.J. Reid
Anaerobic Research Unit
Department of Microbiology
University of Cape Town

ABSTRACT

The regulation of nitrogen (N) metabolism is being investigated in the Gram positive anaerobic bacterium *Clostridium acetobutylicum,* which has been utilized in industrial fermentations to produce acetone, butanol and ethanol. The *C. acetobutylicum* glutamine synthetase (GS) *glnA* gene region originally cloned in *Escherichia coli,* is characterized by the *glnA* structural gene, the upstream promoter sequences P_1 and P_2, a downstream DNA sequence complementary to the 5'end of the *glnA* gene and the downstream promoter P_3. Transcription of the *glnA* gene is controlled by the upstream promoters P_1 and P_2. Transcription from the downstream promoter P_3 in the opposite orientation of the *glnA* gene was demonstrated to express the 43-base *glnA* antisense (AS) RNA in *E. coli* and *C. acetobutylicum* cells. The expression of GS activity or the *glnA* AS RNA were not regulated by N in the heterologous *E. coli* host, but the expression of the antisense RNA in these cells was associated with decreased levels of GS activity. The regulation of the *glnA* gene expression was studied in *C. acetobutylicum* cells after suitable media for the growth of this bacterium was developed. *C. acetobutylicum* GS activity, the transcription of *glnA* mRNA and the *glnA* AS RNA were regulated by N. In cells grown in N-rich medium GS activity and *glnA* mRNA were repressed. Repression ratios for GS activity varied from 1.6 to 9.0 depending on the sampling time. The relative number of *glnA* transcripts was approximately 25%-28% lower in cells grown in N-limiting medium. The expression of the *glnA* AS RNA was differentially regulated relative to the GS activity and *glnA* mRNA levels. The *glnA* AS RNA was repressed in N-limiting medium and induced in N-rich medium. The relative number of AS RNA

transcripts was approximately 1.5-fold in excess of *glnA* mRNA transcripts under conditions that repressed GS activity. Under conditions that induced GS activity, *glnA* mRNA transcripts exceeded AS RNA transcripts. Since differential regulation by N levels of the *glnA* AS RNA expression relative to the *glnA* gene was demonstrated in *C. acetobutylicum*, additional regulatory element(s) affecting the *C. acetobutylicum glnA* system were investigated. *C. acetobutylicum* gene libraries were cotransformed *in trans* with an in-frame *glnA-lacZ* fusion construct and plasmids from the *C. acetobutylicum* gene libraries were tested for β-galactosidase expression. No alteration of the *lacZ* gene expression was detected in the cotransformed colonies. However, DNA sequencing of the region situated downstream of the *C. acetobutylicum glnA* gene revealed the presence of an open reading frame (ORF) located 199 to 766bp from the 3'end of the *glnA* structural gene. The *glnA* AS coding region is located on the putative ribosome binding site and the 5'region of this *C. acetobutylicum* ORF. The protein encoded by this ORF showed 30% similarity with the carboxy terminus of the *Pseudomonas aeruginosa* aliphatic amidase regulator encoded by the *amiE* gene. The amino terminus of this protein also has 28% and 26% similarity with the amino terminal region of the DegU and CheB response regulators, respectively. These regulators belong to the family of the response regulators involving two component signal transduction systems, suggesting that the protein encoded by this ORF may play a role in the mechanism of regulation of the *C. acetobutylicum glnA* gene in response to nitrogen.

ABBREVIATIONS

A	adenine
A_{600}	absorbance at 600 nm
aa	amino acid(s)
ABE	acetone-butanol-ethanol
Act	activity (enzyme)
Ap	ampicillin
ATP	adenosine 5'-triphosphate
AS RNA	antisense RNA
bp	bp
BSA	bovine serum albumine
C	cytosine
cAMP	adenosine-3',5'-cyclic monophosphate
CBM	Clostridial Basal Medium
cDNA	complementary DNA
Ci	Curie
Cm	Chloramphenicol
CsCl	caesium chloride
C-terminal	carboxy-terminal
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
ds	double stranded
DTT	1,4-dithio-L-threitol
EDTA	ethylenediaminetetra-acetic acid
EtBr	ethidium bromide
EtOH	ethanol
g	gram
g	gravitational force
G	guanine
GDH	glutamate dehydrogenase
gln	glutamine
glu	glutamate
GOGAT	glutamine-oxoglutarate amido transferase
GS	glutamine synthetase
GSMM	glucose-salts-mineral-medium
h	hour(s)
IPTG	isopropyl-β-D-thiogalactopyranoside
kd	kilo dalton
kb	kilobase
Km	kanamycine
L	liter
LB	Luria-Bertani (medium)

min	minute(s)
M_r	relative molecular mass
mRNA	messenger RNA
mg	milligram
nt	nucleotide(s)
N	nitrogen
N-terminal	amino terminal
ORF	open reading frame
ori	origin of replication
p	plasmid
$P_{1,2,3}$	promoter 1,2,3
PEG	polyethylene glycol
RBS	ribosome binding site
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolution per min
S	(superscript) sensitivity
s	second
SD	Shine-Dalgarno sequence
SDS	sodium dodecyl sulphate
ss	single stranded
T	thymine
TAE	Tris-acetate EDTA buffer
TBE	Tris-borate EDTA buffer
Tc	tetracycline
Tris	Tris (hydroxymethyl) aminomethane
Trp	tryptophane
Tyr	tyrosine
U	units
U	uracil
UV	ultraviolet (light)
v/v	volume to volume
w/v	weight to volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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CHAPTER ONE

1. GENERAL INTRODUCTION AND LITERATURE REVIEW

The biotechnological importance of *Clostridium acetobutylicum* has been acknowledged since the beginning of the century when it was initially used for the acetone-butanol-ethanol (ABE) fermentation. Today, there is a revival of interest in this microorganism for the production of solvents and organic acids from renewable resources. A better understanding of the molecular biology and biochemical aspects for the genetic manipulation of *C. acetobutylicum* will considerably improve its biotechnological exploitation (Woods, 1993).

1.1 Acetone-butanol-ethanol fermentation

The ABE fermentation by *C. acetobutylicum* has been reviewed by Rogers, 1986; Mc Neil and Kristiansen, 1985; Jones and Woods, 1986a, Woods, 1993. The microorganism involved, *C. acetobutylicum*, is a strictly anaerobic, Gram- positive spore-forming bacterium (O'Brien and Morris, 1971) which can be isolated from various habitats including soil, corn and potatoes (Volesky et al., 1981).

acetobutylicum can utilize a variety of carbohydrate sources which are initially fermented to acetic and butyric acid intermediates, and subsequently to acetone, and butanol. A small amount of ethanol is also produced during the fermentation. *C. acetobutylicum* undergoes physiological (Prescott and Dunn, 1959), morphological (Jones et al., 1982) and biochemical (Duong et al., 1983; Zeikus, 1983) changes during the course of the fermentation process (Jones and Woods, 1986a; Rogers, 1986).

Two characteristic physiological phases are predominant in the conventional batch ABE fermentation (Gottschal and Morris, 1981a; Jones et al., 1982). The first phase, in which there is a decrease in the pH due to the conversion of carbohydrates into acetic and butyric acid, is defined as the acidogenic phase. In this phase, the ratio of acetic and butyric acid seems to be related to the respective amounts of acetone and butanol produced during the second, or solventogenic, growth phase (Martin et al. 1983).

The solventogenic phase involves a switch in the carbon flow from the acid-producing pathways to the solvent-producing pathways. One of the environmental triggers associated with the switch involves the increase in acids and the attainment of the "pH break point" (Jones et al., 1982). The cells then utilize the acids and remaining carbohydrates to generate the solvents acetone, butanol and ethanol as the final products.

The biochemical pathways and the fermentation profiles in the conversion of carbohydrates to acids, gases and solvents by *C. acetobutylicum* have been extensively documented (Dürre et al., 1987; Matta-El-Ammouri et al., 1987; Papoutsakis and Bennet, 1993). The energy metabolism (Haggstrom, 1985) and the biochemical pathways involved in the fermentation by *C. acetobutylicum* have also been described (Rogers, 1986; Jones and Woods, 1986a, 1989).

1.2 Studies on the molecular biology of *C. acetobutylicum*

In recent years recombinant DNA technology has become an important tool for the analysis of *C. acetobutylicum* metabolic and genetic systems at the molecular level. Studies have been directed towards the genetic manipulation of this microorganism for improved metabolic and industrial capabilities. The development of a genetic system has required the use of a suitable plasmid

vector containing a selectable genetic marker for the transfer of DNA into *C. acetobutylicum*, and also for the cloning and characterization of central metabolic genes.

1.2.1. Development of a genetic system for *C. acetobutylicum*

1.2.1.1. Gene transfer. One impediment for the transfer of DNA into *C. acetobutylicum* by protoplast fusion, transformation and transfection, has been the presence of high levels of extracellular and cell bound deoxyribonuclease activity (Jones and Woods, 1986b). However, early studies demonstrated transfection of *C. acetobutylicum* protoplasts with phage DNA (Reid et al., 1983). High transformation frequencies of *C. acetobutylicum* have been obtained when the plasmids pVA1, pVA677, and phage HM3 DNA were utilized (Podvin et al., 1988).

The initial system developed for the conjugative transfer of DNA into *C. acetobutylicum* was dependent on the broad host range streptococcal vector pAMβ1 (Reysset and Sebald, 1985). This vector was shown to be transferred to *C. acetobutylicum* from a number of donors, including *Streptococcus lactis, Streptococcus faecalis, Bacillus subtilis,* at relatively high frequencies. Other broad host range vectors such as pIP501, pJH4, and pVA797 have been transferred into *C. acetobutylicum* by conjugation (Young et al., 1989a, 1989b).

The transfer of conjugal transposons to a variety of Gram-positive hosts (Clewell and Gawron-Burke, 1986) including *C. acetobutylicum* (Young et al., 1989a, 1989b) has demonstrated their broad host range. The use of transposons in *E. coli* has been applied in a variety of genetic studies including the identification of promoters, gene cloning, analysis of export mechanisms, genetic mapping, and the creation of specific and polar mutations (de Bruijn and Lupski, 1984).

Bertram et al. (1990) were able to generate three types of mutants of *C. acetobutylicum* DSM 792 by utilizing the Tn*916* conjugation/mutagenesis system.

The application of electroporation in the transformation of a variety of Gram-positive bacteria has been very effective (Lucansky et al., 1988). *C. acetobutylicum* NCIMB8052 has been transformed with an efficiency of $3x10^3$ to $6x10^3$ transformants per mg DNA (Oultram et al., 1988; Lee et al., 1992), although the presence of restriction systems initially prevented the electrotransformation of *C. acetobutylicum* ATCC824 and P262 (Lee et al., 1992).

1.2.1.2. Cloning vectors. The lack of suitable genetic markers present in the native plasmids from the genus *Clostridium* (Minton and Thompson, 1990) has made it necessary to design and construct vectors carrying antibiotic genes and replicons able to function in *C. acetobutylicum* (Truffaut et al., 1989; Minton and Oultram, 1988; Minton et al., 1990a, 1990b; Yoshino et al., 1990; Lee et al., 1992).

Initial vectors (i.e. pMTL20E, pMTL20T and pMTL20C) were constructed from pUC-like cloning vectors containing antibiotic resistance genes such as Erythromycin (Em), Tetracycline (Tc), and Chloramphenicol (Cm), a multiple cloning site within the lacZ' region and an *E. coli* origin of replication. pAMβ1 was utilized for later constructs due to its structural stability. The replication region of this plasmid has been analyzed and subcloned into the pasmids pMTL20E, pMTL20T and pMTL20C to yield the vectors pMTL500E, pMTL500T and pMTL500C (Swinfield et al., 1990; Minton et al., 1990b). The vector pMTL500E was shown to replicate either in *C. acetobutylicum* or *B. subtilis*. In the absence of antibiotic selective pressure some of these constructs have exhibited varying degrees of segregational instability (Truffaut et al., 1989; Yoshino et al., 1990).

Another construct based on the heterologous plasmid pAMβ1 is the *E. coli*-*C. acetobutylicum* shuttle vector, pSYL9, which was obtained as a fusion of pBR322 (Bolivar et al., 1977) and pIL252, a small derivative of pAMβ1. This shuttle vector carries ampicillin and erythromycin resistant markers, and transformed both *E. coli* DB11 and *C. acetobutylicum* NCIMB8052 (Lee et al., 1992). Although vector transfer systems are being developed, so far no reporter genes are available to study regulation in the homologous host.

1.2.2. Sporulation and stationary phase events

acetobutylicum and *B. subtilis* are both Gram-positive, sporeforming, soil bacteria. Endospore formation in *B. subtilis* has been extensively characterized with respect to the metabolic and genetic events taking place during sporulation (Sonenshein, 1985, Stragier and Losick, 1990). In this bacterium, two stages can be recognized during the process of endospore formation. The initiation stage, where cells are able to sense environmental changes that do not offer enough support for continuous rapid growth, and the differentiation stage, where sporulation gene products are sequentially expressed in a genetically defined manner.

It has been shown that the transfer of *B. subtilis* cells from a rapidly growing culture to a nutritionally poor medium initiates spore formation (Sterlini and Mandelstam, 1969; Losick et al., 1986; Sonenshein, 1989). In contrast, *C. acetobutylicum* cells do not respond via the same sporulation inducing events when they are grown under starvation conditions (Woods and Jones, 1986). In *C. acetobutylicum* cells, glucose or ammonia limitation are not sufficient for the induction of solventogenesis or sporulation (Long et al., 1984a). *C. acetobutylicum* sporulates only if growth is limiting in the presence of an excess of nitrogen (N) and carbon sources (Jones and Woods, 1986a).

In *C. acetobutylicum*, sporulation and solventogenesis appear to share the same inducing factors, such as a decrease in the pH and acid end-product accumulation. Furthermore, a spore-forming deficiency has been correlated with a lack of solvent production, suggesting the existence of a common switch for both processes (Gottschal and Morris, 1981a). However, the formation of spores is not necessarily required for the production of solvents. It has been shown that solvent production was unaffected in asporogeneous *C. acetobutylicum* mutants blocked before (SPO 0) or after (SPO II-VII) forespore septum initiation (Jones et al., 1982; Long et al., 1984b).

The role of glutamine synthetase (GS:EC 6.3.1.2.) in sporulation has been analyzed as in *Bacillus* strains this enzyme is central to both N and carbon metabolism. *Bacillus megaterium* and *B. subtilis* glutamine (gln) auxotrophs have been isolated with reduced levels of GS. These bacteria were unable to sporulate. gln-independent revertants reestablished their normal sporulation properties and GS activity (Reysset et al., 1978). In *Bacillus licheniformis*, the enzymes or metabolites of the N assimilation pathway do not seem to take part in the sporulation control signal (Schreier et al., 1981). In *B. subtilis* grown under conditions of carbon or N limitation which induce sporulation, GS activity levels remained unaltered. Although, in a glucose and gln medium GS activity increased upon gln deprivation (Pan and Coote, 1979). A N fixing facultative anaerobe, *Bacillus polymyxa*, showed a lower GS activity as well as a reduced intracellular glutamate (glu) pool during the onset of sporulation, and the specific inhibition of GS by L-methionine sulfoximine led to the induction of sporulation (Kantengwa and Ohja, 1985; Kantengwa and Ohja, 1986). Since N metabolism appears to be important for differentiation, the enzymes involved and their regulation have been studied to elucidate the control mechanisms implicated in solventogenesis and sporulation in *C. acetobutylicum*.

1.3. Nitrogen metabolism

1.3.1. Sensory systems. Prokaryote cells display a remarkable ability to utilize the information regarding fluctuations of metabolites in the surroundings and to elicit appropriate adaptive responses. They react to the environment by sensing extracellular and intracellular concentrations, or fluxes between these pools and reporting via regulatory proteins. A great variety of signal transduction proteins have been shown to participate in regulatory processes involved in the stimulus-response sensory systems (Ninfa et al., 1988; Stock et al., 1988; Forst et al., 1989; Bourret et al., 1990, 1991; Kofoid and Parkinson, 1988, 1992; Parkinson, 1993).

These signaling proteins communicate through the interaction of two specialized and characteristic domains: the transmitter and the receiver domains. There are a variety of signaling processes in which these transmitter and receiver domains establish communication. They include pathogenesis (Miller et al., 1989; Winans, 1991), symbiosis (Gottfert et al., 1990), carbon metabolism (Island et al., 1992), N metabolism (Keener and Kustu, 1988), electron transfer (Lin and Iuchi, 1991), phosphorylation (Wanner, 1992), physiological responses to stress-induced differentiation, such as sporulation (Burbulys, et al., 1991), and physiological responses to changes in the medium osmolarity (Mizuno, 1991).

Basically, signaling systems can involve two protein components: a "sensor", often situated in the cytoplasmic membrane, that checks some environmental parameter; and a cytoplasmic "response regulator" that mediates changes in gene expression in response to sensor signals (Nixon et al., 1986; Ronson et al., 1987) (Fig.1.1). Sensor proteins contain a carboxy-terminal transmitter domain of approximately 240 residues. Response regulators are characterized by an amino-

terminal receiver domain of about 120 amino acids (aa). These domains act in association with other "input" and "output" domains. In a sensor protein, the input domain regulates transmitter activity. The receiver domain activates or inhibits the related output domain according to the signal obtained from the transmitter. Signaling between the transmitter and the receiver involves modification by phosphorylation or dephosphorylation. An autokinase transmitter activity has been demonstrated to phosphorylate a histidine residue by using ATP phosphoryl groups. The phosphoryl groups attached to the histidine residue are subsequently transferred to an aspartate residue located in the receiver. Receiver phosphorylation in turn, regulates the activity of its associated output domain. Regulatory responses are switched off when the receiver is dephosphorylated. This is achieved by a number of specific mechanisms to avoid crosstalk from other signaling pathways.

Two-component signaling systems are simple and uncommon. In most other systems multiple components have been shown to participate in association with regulatory circuits, feedback loops or other complex sensory mechanisms.

One of the best understood regulatory systems in which signal transduction mechanisms have been demonstrated is the control of gene expression in response to the intracellular balance between carbon and N metabolism (Magasanik and Neidhart, 1987; Fisher and Sonenshein, 1991).

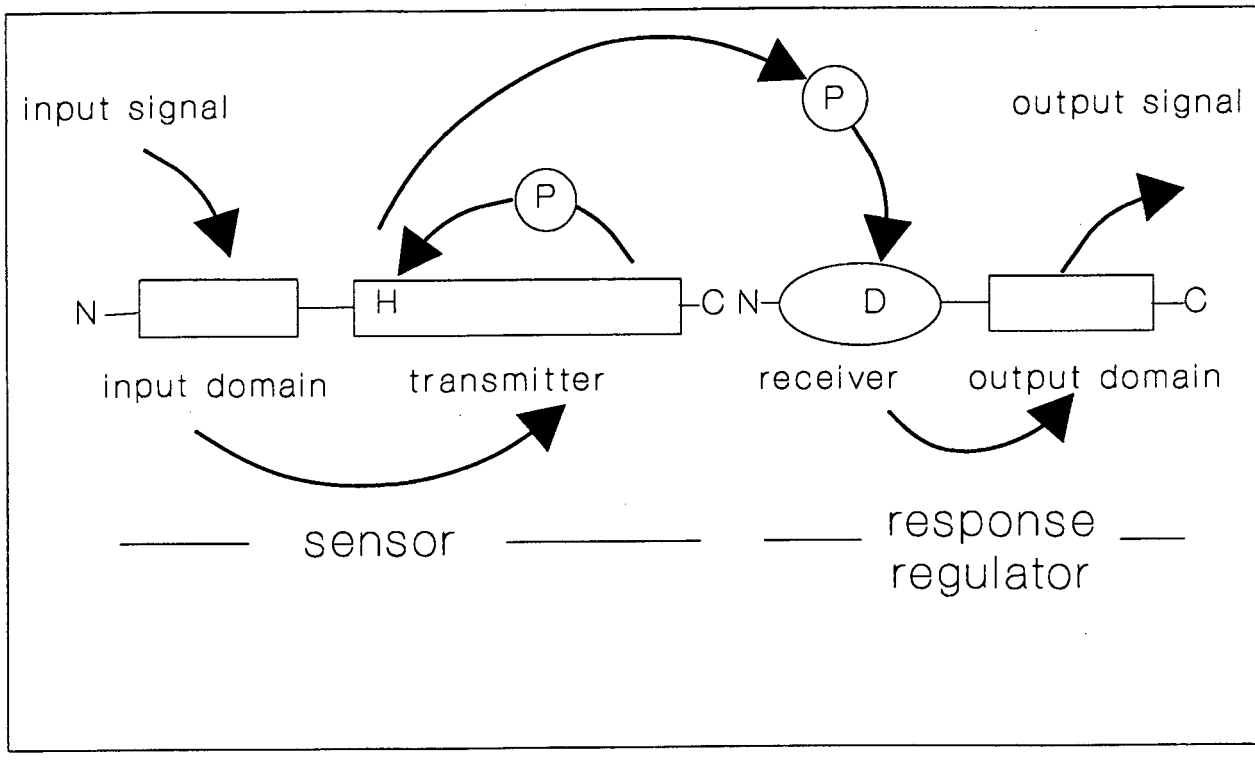

Fig. 1.1. Two component system sensor-regulator mechanism. A signal is received by the input domain from the sensor protein. This signal is forwarded to the sensor-transmitter domain, which has autokinase activity. A histidine residue from the transmitter domain receives a phosphate group. This phosphate group is transfered to an aspartate residue situated in the receiver domain of the response regulator. The phosphorylation of the receiver domain regulates the activity of the output domain, which triggers the regulatory response via an output signal (after Kofoid and Parkinson, 1992).

1.3.2. Nitrogen assimilation. The search, fixation, transport, storage, and incorporation of N in diverse chemical forms represent some of the systems utilized by bacteria to maintain a suitable balance in N metabolism. Reduced N, as found in ammonia ions, amino or amido groups is the form utilized in N metabolism. Nevertheless, for most microorganisms the preferred N source is ammonia, and often inorganic compounds like histidine, arginine and proline are converted to ammonia through enzymatic reactions.

The precise set of pathways utilized to execute these reactions is distinctive for each organism. However, under conditions in which ammonia or ammonium ion is limiting, this N source is incorporated into gln as a metabolic intermediate. When ammonia is available in excess it is metabolized into glu, gln and asparagine.

The aa gln and glu are the main metabolic intermediates in the biosynthesis of N compounds. The role of gln is to supply N for the synthesis of nucleotides such as purines and pyrimidines, amino sugars, other aa like histidine, tryptophan, asparagine, and cofactors such as NAD and p-aminobenzoate. glu is also utilized in the biosynthesis of many other aa.

The metabolic pathways by which N is assimilated through ammonia, gln and glu have been thoroughly studied in the Gram negative enteric bacteria (reviewed by Tyler, 1978; Magasanik, 1982; Kustu et al., 1986; Magasanik and Neidhart, 1987; Reitzer and Magasanik, 1987; Schreier, 1992).

The three key enzymes playing a pivotal role in this process are GS, glutamate synthase (GOGAT:EC 1.4.1.13) and glutamate dehydrogenase (GDH:EC 1.4.1.4). They catalyze the following reactions:

$$NH3 + \text{glutamate} + ATP \xrightarrow{GS} \text{glutamine} + ADP + Pi$$

$$\text{glutamine} + \text{2-ketoglutarate} + NADPH + H^+ \xrightarrow{GOGAT} 2\text{-glutamate} + NADP^+$$

$$NH_3 + \text{2-ketoglutarate} + NADPH + H^+ \xrightarrow{GDH} \text{glutamate} + NADP^+$$

1.3.3. Glutamine synthetase. GS is considered to be the most important enzyme in the N metabolism of bacteria. The GS pathway represents the only known route by which gln can be synthesized. Under N limiting conditions GS introduces N from ammonia into gln, which is used in the synthesis of various important metabolites. Both, the synthesis of GS and its catalytic activity are controlled by very accurate regulatory systems acting in response to N availability, which is determined by the intracellular ratio of gln to α-ketoglutarate.

1.3.3.1 GS structure. Two types of GS enzymes have been previously characterized as GSI and GSII type enzymes. Prokaryotes and eukaryotes were once thought to synthesize different GS enzymes: GSI for the former and GSII for the latter. It is now known that GSI exists in the *Enterobacteriaceae* (Streicher and Tyler, 1980; Almassy et al., 1986), *Vibrio* (Bodasing et al., 1985), *Thiobacillus* (Barros et al., 1986), *Bacillus* (Donohue et al., 1981; Deuel et al., 1970; Hubbard and Stadtman., 1967; Wedler et al., 1980), *Clostridium* (Usdin et al., 1986); Janssen et al., 1988), and *Streptomycetaceae* (Paress and Streicher, 1985; Fisher et al., 1989). GSII is present in eukaryotes, and coexists with GSI in the genera from the *Rhizobiaceae, Frankiaceae,* and *Streptomycetaceae* (Carlson and Chelm, 1986; Darrow and Knotts, 1977; Filser et al., 1986; Rochefort and Benson, 1990; Kumada et al., 1990).

A recent phylogenetic analysis was performed on GSI and GSII aa sequences derived from both prokaryote and eukaryote gene sequences. It was shown that both GSI and GSII gene structures came from a single ancestral gene. Gene duplication of this ancestral gene resulted in the GSI and GSII genes. The duplication event was estimated to have preceded the divergence of eukaryotes and prokaryotes by more than 1000 million years. This study has suggested that GS encoding genes are some of the oldest functional genes in the history of gene evolution (Kumada et al., 1993).

An atypical GS was identified in two anaerobes, *Bacteroides fragilis* (Southern et al., 1986, 1987; Hill et al., 1989) and *Butyrivibrio fibrisolvens* (Goodman et al., 1993). These GS enzymes show significant homology to each other, but differ markedly from GSI and GSII, which indicates that they represent a new GSIII type of structure. Structural and regulatory aspects of GSI, GSII and GSIII types have been reviewed by Woods and Reid, 1993.

Characteristic structural features of the GSI type enzyme are: a dodecamer organized in a toroid structure formed by two rings of 6 subunits, each one consisting of 444 to 474 aa (Janssen et al., 1988), and a total M_r of 600 kd (Streicher and Tyler, 1980). GSI catalytic activity is dependent on the presence of two manganese cations associated with the active site. An X-ray crystallographic atomic model has been proposed for the *Salmonella typhimurium* GSI (Almassy et al., 1986; Yamashita et al., 1989).

An unusual feature of GSI is the position of the active site between heterologous subunit interfaces. The structure of the active site is formed by six β-strands of the large C-domain and two β–strands from the Trp-57 loop of the smaller terminal domain of the next subunit (Almassy et al., 1986). An analysis of the central space of the GSI subunits elucidated the presence of a loop structure located between aa 154 and 177. Protease sensitivity was demonstrated since a

number of specific protease activities affected this region (Dautry-Varsat et al, 1979; Monroe et al., 1984, 1985; Almassy et al., 1986). An exception to the GSI type is represented by *B. subtilis* (Strauch et al., 1988) and *C. acetobutylicum* GSI (Janssen et al., 1988) in which this 26 aa region is not present. The GS of these bacteria and other Gram-positive bacteria, such as *Streptomyces coelicolor*, are characterized by the Trp residue being replaced by a Phe residue (Strauch et al., 1988; Wray and Fisher, 1988; Janssen et al., 1988).

A further classification of GSI enzymes can be done according to modification by adenylylation. Both *Clostridium* GSI (Usdin et al., 1986) and *Bacillus* GSI (Schreier et al., 1985) lack this adenylylation system, although *S. coelicolor* GSI (Wray and Fisher, 1988) and GSI enzymes from the *Enterobacteriaceae, Vibrio* (Bodasing et al., 1985), and *Thiobacillus* (Barros et al., 1986) are all regulated by adenylylation.

The region surrounding the Tyr residue 401, the adenylylation site for *E.coli* GSI, (Shapiro and Stadtman, 1968; Heinrikson and Kingdon, 1971), is highly conserved among GSI enzymes that are modified by adenylylation. The conserved position of the Tyr residue is shared by GSI enzymes from *S. typhimurium* (Janson et al., 1986), *T. ferrooxidans* (Rawlings et al., 1987), *Streptomyces* (Fisher and Wray, 1989), and *Anabaena* (Tumer et al., 1983; Fisher et al., 1981).

The GSII enzyme structure is characterized by a heat labile octamer consisting of identical subunits arranged in two parallel discs of 4 subunits (Carlson and Chelm, 1986). The GSII subunits are smaller, 329 to 373 aa (Gebhardt et al., 1986: Hayward et al., 1986; Tischer et al., 1986), and appear to be missing the adenylylation site and the C-terminal end when compared to the GSI subunits. The similarity among GSI and GSII subunits is not very significant (approximately 15%) (Almassy et al., 1986; Rawlings et al., 1987). However, there are five highly conserved regions in which this similarity among GSI and GSII is

distributed. One of these regions, region I, is involved in the formation of the active site with the participation of the Trp-57 loop residue. Regions II and V represent β-strands in association with manganese cations.

The structure of the GSIII type of enzyme has been described as a hexamer of large identical subunits containing 729 aa. Each subunit is approximately 270 or 400 aa longer than the subunits of the GSI and GSII enzymes, respectively. The conserved 18 aa region involved in adenylylation is not present in GSIII. A Val residue instead of the Trp-57 loop residue is present in GSIII.

1.3.3.2. Regulation of GSI expression. Studies on the expression of the *Enterobacteriaceae glnA* gene have shown that regulatory mechanisms are interlinked and that the regulation of GS enzymes is accomplished by feedback inhibition and covalent modification of the catalytic activity. These mechanisms involve a complex set of reactions which constitute a "bicyclic cascade" generating a fast and accurate response relative to the N availability (Fig. 1.2) (Tyler, 1978; Magasanik, 1982; Magasanik and Neidhardt, 1987; Reitzer and Magasanik, 1987; Magasanik, 1988; Stock et al., 1988).

GS is expressed under N-limited growth conditions, and is positively regulated at the level of activity. When N sources are abundant, the GS dodecamer becomes adenylylated. One adenyl group is covalently attached to a tyrosine residue in each subunit. This adenylylation inactivates each individual subunit, so the overall GS activity decreases in relation to the number of adenylylated subunits. Conversely, under N-limiting conditions, GS is deadenylylated representing a relatively high activity enzyme form. The adenylylation-deadenylylation reactions are catalyzed by an adenylyltransferase enzyme encoded by the *glnE* gene, the activity of which is partially controlled by the *glnB* encoded P_{II} regulator. P_{II} forms a common link between the regulation of GS

activity by adenylylation and the regulation of *glnA* expression by the Ntr system of transcriptional regulation (Garcia and Rhee, 1983; Bueno et al., 1985; Son and Rhee, 1987). P_{II} itself is controlled by covalent modification by a uridylyltransferase, which represents a bifunctional polypeptide able to add or remove uridylyl groups from the P_{II} protein. Therefore, uridylyltransferase dictates the direction of adenylylation or deadenylylation of each GS subunit according to the N status of the cell (Stadtman et al., 1980; reviewed in Reitzer and Magasanik, 1987). During N-rich conditions, deuridylylated P_{II} stimulates the adenylylation reaction of the adenylyltransferase with a subsequent inactivation of GS. Under N-limiting conditions, uridylylated P_{II} stimulates the deadenylylation reaction of adenylyltransferase, which increases GS activity.

Another level of regulation consists of the cumulative feedback inhibition by the metabolic products of gln. The adenylylated form of GS is partially inhibited by L-alanine, glycine, histidine, tryptophan, CTP, AMP, carbamyl phosphate, and glucosamine-6-phosphate. This inhibitory effect is cumulative and all these compounds are able to inhibit GS totally (Stadtman and Ginsburg, 1974).

The genetic analysis of the *glnA* region in enterobacteria identified the components of the *glnALG* or *glnAntrBC* operon (Fig. 1.2), which is at the centre of the global Ntr N regulation system. The expression of this operon has been

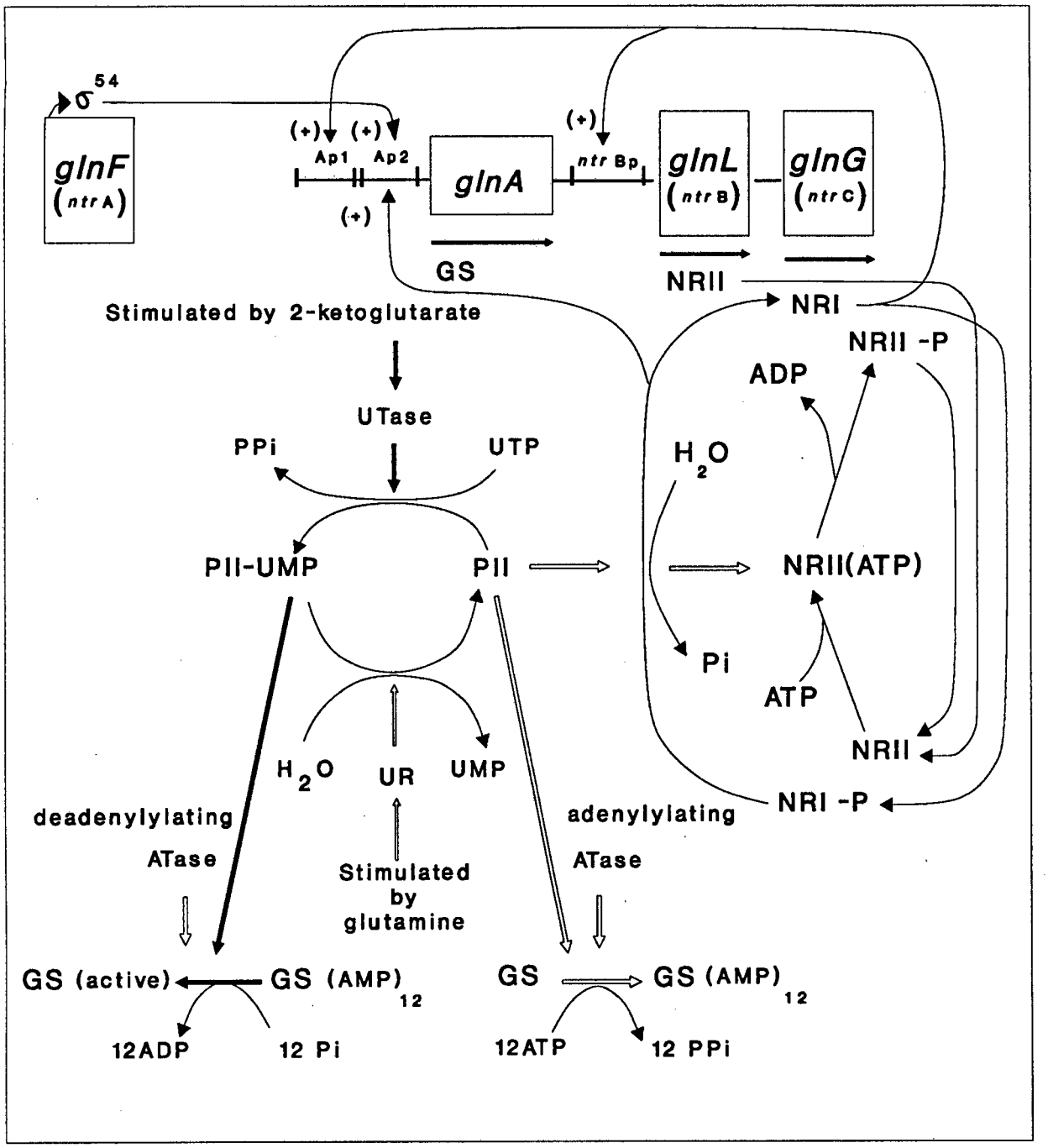

Fig. 1.2. Regulatory mechanism of GSI expression in Enterobacteria. Different levels of control are shown to be integrated in coordinated systems which regulate the expression of the *glnA* gene and the GS activity (after Reid and Woods, 1993).

extensively studied in the enterobacteria (Reitzer and Magasanik, 1985, 1986; Kustu et al., 1986; Keener et al., 1987; Macfarlane and Merrick, 1985, 1987). The products of *glnL* (*ntrB*) and *glnG* (*ntrC*) represent the regulators NR_{II} (NtrB) and NR_I (NtrC), respectively, and the structural *glnA* gene encodes the GS. The operon contains three promoters. Two *glnA* upstream promoters, *glnAp1* and *glnAp2*, 187 and 137 bp upstream, respectively, and a third one, $ntrB_p$ located between the *glnA* and *ntrB* genes. *glnAp1* and $ntrB_p$ are consensus σ70 promoters regulated by proximal elements and are recognized by σ70-RNA polymerase (Eσ70), while *glnAp2* is different and recognized by σ54-RNA polymerase (Eσ54). This specialized sigma factor is encoded by the *ntrA* (*glnF,rpoN*) gene. The σ54 promoter sequences are characterized by GG and GC doublets situated 24 and 12 bp upstream of the transcription start point, respectively. These promoter sequences differ from the σ70 consensus sequences in that they are activated in the presence of enhancer proteins interacting at a certain distance from the σ54 binding site (Kustu et al., 1989; Collado-Vides et al., 1991; Buck and Cannon, 1992). Under N excess conditions *glnAp1* and $ntrB_p$ promoters control low transcription levels of: *glnA*, *ntrC* and *ntrB* products, maintaining a basal level of GS, NR_I and NR_{II}, respectively. Under conditions of carbon limitation, the catabolite gene-activator protein (CAP) and cyclic AMP (cAMP) bind to a site upstream of *glnAp1* and further stimulate transcription from the *glnAp1* promoter region (Magasanik and Niedhardt, 1987). Approximately three out of every four transcripts from *glnAp1* terminate at a *rho*-independent terminator located in the region upstream of $ntrB_p$. NR_I represses *glnAp1* and $ntrB_p$ by binding to the -35 upstream region site of *glnAp1* and to the -10 upstream region of $ntrB_p$. The NR_I binding site is characterized by the nucleotide sequence $GCACN_5TGGTGC$. NR_I also represses the expression of *glnAp2* and there are five NR_I binding sites upstream of *glnAp2*. NR_I binds with high affinity to two sites, NR-1 and NR-2, located in the region between -140 and -110 bp upstream of

the initiation of transcription site. Under N-limiting conditions the phosphorylated state of the NRI protein (NR_I-P) immediately activates the initiation of transcription from *glnAp2*, as Eσ54 is permanently bound to *glnAp2* in an inactive closed promoter complex irrespective of the N-status (Reitzer et al., 1987). The phosphorylated form of NR_I, therefore stimulates this closed promoter complex to an open and active form. It is the activation of NR_I by NR_{II}-mediated phosphorylation that ultimately results in the activation of *glnA* transcription, and represents the second component of the "bicyclic cascade" regulation of GS in response to the N status.

NR_{II} phosphorylates NR_I rendering it active by a reversible phosphorylation reaction (Ninfa and Magasanik, 1986; Ninfa et al., 1986). This reaction is performed in two steps. First, the γ-phosphate group of ATP is incorporated into a histidine residue of NR_{II}. The histidine phosphate group is then transferred to an aspartate residue of NR_I by a reaction with NR_{II}.

Under N-limiting conditions, the phosphorylated form of NR_I activates transcription from *glnAp2* increasing the GS and NR_I-P levels, while the gln/2-ketoglutarate ratio is still low and P_{II} remains urydylylated. Under N excess conditions P_{II} becomes deurydylylated signaling the dephosphorylation of NR_I-P, which is then unable to activate transcription from *glnAp2*.

In the Gram-positive *B. subtilis* GSI, the rapid modification by adenylylation characteristic of the *Enterobacteriaceae* is not present, and no other post-translational control mechanisms have been detected. Although combinations of various metabolites are able to inhibit *B. subtilis* GS activity, regulation at the level of transcription appears to be the most important regulatory mechanism (Fisher and Sonenshein, 1984; Reitzer and Magasanik, 1987).

Schreier (1992) has recently reviewed the regulation of *B. subtilis* GSI, which displays a distinct autoregulatory control of its own synthesis. Early studies based on deletion derivatives of *B. subtilis glnA* supported the initial autoregulatory hypothesis of Dean et al. (1977). The involvement of GS enzyme itself in the regulation of gene expression from the *glnA* promoter has been demonstrated (Schreier et al., 1985). *B. subtilis* strains carrying fusions of the *glnA* promoter region to the *E. coli lacZ* gene showed negative regulation of β-galactosidase expression, when the *glnA* DNA region was present *in trans.* Experiments in which mutations were introduced in the *glnA* gene altered this negative regulation. Therefore, studies have supported the idea that *B. subtilis* GS participates in the regulation of its own synthesis at the level of transcription (Schreier and Sonenshein, 1986). A further analysis of the DNA sequence of the *glnA* upstream region, situated between the *glnA* promoter and the start of the structural *glnA* gene, revealed the presence of the *glnR* gene coding for a 135 aa protein regulator (GlnR), which acts in conjunction with GS to negatively regulate *glnA* transcription (Schreier et al., 1989) (Fig. 1.3).

Both *glnA* and *glnR* genes form part of the *glnRA* operon. Experiments have shown evidence of GlnR acting as a DNA binding repressor in this operon. The GlnR protein structure contains a α-helix-turn-α-helix motif. GlnR forms a dimer and inhibits transcription by binding specifically to two operator sequences, $glnRAo_1$ and $glnRAo_2$. These two operator sequences are located within a region adjacent to the *glnA* promoter region. *In vivo,* GlnR interacts with these operator sequences under N excess conditions (Schreier et al., 1989; Schreier et al., 1991; Gutowski and Schreier, 1992).

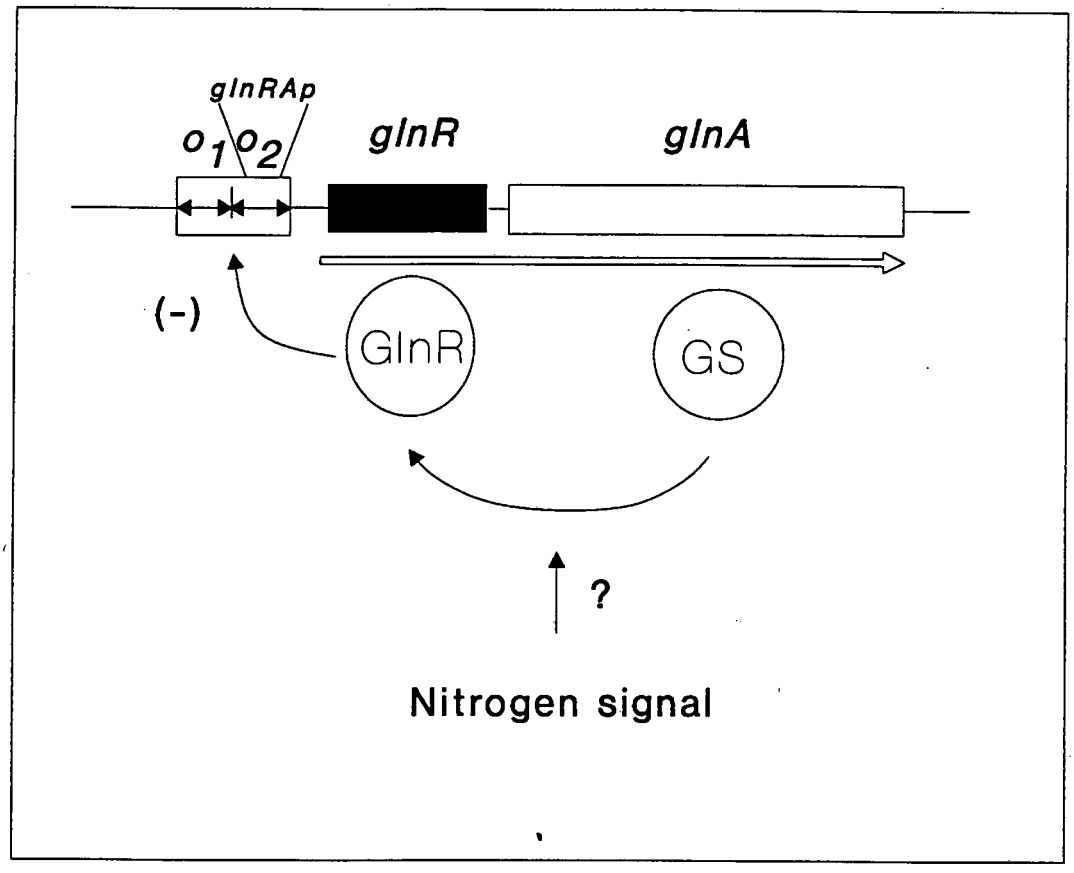

Fig. 1.3. Regulation of the *B. subtilis glnA* gene. The *glnRA* operon region is charaterized by the presence of the structural *glnA* gene, a *glnR* gene, two operator sequences and a *glnRA* promoter sequence located in the region upstream of *glnA*. The *glnR* gene encodes a protein regulator, which in conjunction with GS would sense a N signal. Under N excess conditions GlnR interacts with the operator sequences $glnRo_1$ and $glnRo_2$ inhibiting transcription (after Schreier H.J, 1992).

The mechanism of regulation of GlnR activity by N has not been established yet. Post-translational modification does not play a role in the control of GlnR activity by N. However, GlnR regulation may be mediated by some N-dependent factor(s) somehow related to GS activity. It has been suggested that GS would sense the availability of a fundamental N metabolite and signal GlnR by a certain type of interaction. Mutagenesis studies on the GlnR protein indicated that the carboxy terminal region was involved in sensing N conditions. Thus, it was shown that deletion of the last seven aa residues blocks derepression under N limitation. The mechanism of interaction between GlnR and GS, or the N sensory systems in which both appear to play a role, have not been elucidated as yet (Fisher and Sonenshein, 1991; Schreier, 1992; Gutowski and Schreier, 1992). Other protein(s) in addition to GlnR appear to be interacting with the *glnRA* regulatory region (Schreier, 1992). Under N-limiting conditions, the mutation of certain bases corresponding to the $glnRAo_2$ operator sequence suggested the participation of extra regulatory factors in the control of *glnRA* (Gutowski and Schreier, 1992). No global N regulatory system has been shown to be involved and gln/2-ketoglutarate ratios do not affect GS activity. The *B. subtilis outB* gene product has been implicated in the regulation of *glnA*. Its sequence shows similarity to an *E. coli* gene product that complements Ntr-like mutations in *Rhodobacter capsulatus*. Furthermore, an *outB81* mutation in the *outB* gene prevents *B. subtilis* cell growth and *glnRA* transcriptional activation in a medium containing N sources that were metabolized slowly (Albertini and Galizzi, 1990).

Evidence indicates that GS plays a role in the regulation of other genes. The mechanism of GS N-regulation appears to be linked to the N regulation of urease and asparaginase. GS was also implicated in the regulation of genes *nrg*-21 and *nrg*-29. In experiments where these two genes were present in *Tn917-lacZ* fusion *B. subtilis* strains, GS was shown to alter the expression of β-galactosidase (Fisher and Sonenshein, 1991; Atkinson and Fisher, 1991).

In the *B. subtilis* levanase operon, the *sigL* gene product, has a very similar aa sequence to σ54. In Gram-positive bacteria there is no evidence that σ54-like proteins have a role in the regulation of N metabolism as in Gram-negative bacteria. Mutations in the *sigL* gene are pleiotrophic. They affect the levanase operon and the metabolism of arginine, ornithine, valine, and isoleucine. The levanase operon is transcribed from a promoter located at positions -12 and -24 upstream of the initiation start point. Experiments have shown that the LevR activator protein has a 200-aa domain similar to the central domains of NifA and NtrC. LevR protein has been demonstrated to interact with a distant site relative to the transcription point (Debarbouille et al., 1991).

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1.4. Regulation of Gene expression by antisense RNA in bacteria

Antisense (AS) RNA is defined as a small RNA sequence that is able to bind to a complementary region of a target RNA and affect its function (Eguchi and Itoh, 1991).

The regulation by AS RNA in bacteria has been extensively studied and reviewed (Green et al., 1986; Inouye and Delihas, 1988; Polisky, 1988; Simons and Kleckner, 1988; Inouye, 1988; Simons, 1988; Takayama and Inouye, 1990; Eguchi and Itoh, 1991). Two recent reviews (Thomas, 1992; Simons, 1993) have been of particular importance in the preparation of this section regarding the regulation by AS RNA. The biological role of trans-acting RNA regulatory molecules was discovered in studies related to the control of replication in bacterial plasmids. However, the AS RNA control mechanism has been found to be present in a variety of other bacterial systems involving transposon, phage and chromosomal gene regulation. A list of the well established naturally occurring AS RNA systems according to the proposed mechanisms of action is given in Table 1.1 (Simons, 1993).

1.4.1. Regulation of plasmid replication

1.4.1.1. RNA processing: ColE1 plasmid. Although plasmids usually utilize certain elements of the host's replication machinery all the plasmids so far analyzed have been shown to encode their own copy number control mechanisms. Regulation by AS RNA was first established during the studies on the replication of the *E. coli* plasmid ColE1 (Lacatena and Cesareni, 1981).

Table 1. Natural Antisense RNA Control in Prokaryotic Organisms

Biological system	Antisense RNA	Target RNA	Target site	Biological process controlled
		RNA processing		
Plasmid ColE1 (several cases)	RNAI	RNAII preprimer	Leader	Replication compatibility
		Ribosome binding		
Transposon IS*10*	RNA-OUT	*tnp* mRNA	RBS	Transposition
E. coli ompF	*micF* RNA	*ompF* mRNA	RBS	Osmoregulation
E. coli dicF	*dicF* RNA	*ftsZ* mRNA	RBS	Cell division
Plasmid R1162	*ct*RNA	*repIA* mRNA	RBS	Replication
Plasmid pSL1	RNAII	*repB* mRNA	RBS	Replication
IncF plasmids	*finP* RNA	*traJ* mRNA	RBS	Conjugation
Phage P22	*sar* RNA	*ant* mRNA	RBS	Antirepression
R1 and F plasmids	*sok* RNA	*mok/hok* mRNA	RBS	Postsegregational killing
Clostridium glnA	p_3 RNA	*glnA* mRNA	RBS	Nitrogen regulation
		Translational Attenuation		
Plasmid R1	*copA* RNA	*repA* mRNA	Leader peptide	Replication compatibility
IncB plasmids	RNAI	RNAII	Leader	Replication
		Transcription Termination		
Plasmid pT181	RNAI/II	*repC* mRNA	5'leader	Replication
E. coli crp	*tic* RNA	*crp* mRNA	5'leader	Catabolite repression
		Transcript Stability		
Phage lambda	OOP RNA	cII mRNA	3'coding region	cII Expression
		Other Cases		
Plasmid R6K	SilencerRNA	ActivatorRNA	Uncertain	Origin activity
Phage lambda	P_{aQ} RNA	*Q* mRNA	RBS	Late gene expression
E. coli sulA	*isf* RNA	*sulA* mRNA	3'Coding region	Uncertain

The period of time in which the interaction between AS RNA and its target RNA occurs is critical. These two molecules must reach optimal concentrations at a certain time to be able to regulate the biological process in which they are involved efficiently (Eguchi and Itoh, 1991). Therefore, a major advantage of RNA (with the exception of tRNA, rRNA and certain stable mRNAs) over protein as a regulator in plasmid replication is its relatively short half life. When these small repressor RNAs are constitutively transcribed from a plasmid DNA template, the change in plasmid copy number could be rapidly sensed, and at certain critical concentrations, the rate of RNA-RNA interaction would determine a rapid inhibition or stimulation of plasmid replication.

The ColE1 *E. coli* plasmid carries a gene encoding the colicin E1 lethal protein. Stable inheritance of this plasmid during random cell division is partly due to a relatively high copy number (15 copies per cell during exponential growth) (Summers and Sherratt, 1984). ColE1 initiation of replication depends exclusively on the binding of a preprimer RNA (RNAII) to the origin of replication, *oriV* (Masukata et al., 1987) and no plasmid encoded proteins have been shown to be required. Elongation by DNA polymerase I from a primer resulting from RNAse H cleavage of RNAII causes the initiation of the synthesis of the leading strand (Itoh and Tomizawa, 1980). The efficiency of primer formation is determined by a mechanism that regulates the association of RNAII to *oriV* (Fig. 1.4).

A key element is RNAI, a 108 nucleotide AS RNA transcribed from the opposite strand to RNAII (Tomizawa et al., 1981). RNAI binds to RNAII altering the structure of RNAII in a way that prevents it from developing a specific secondary structure required for interacting with *oriV* (Masukata and Tomizawa, 1986). To impede the formation of the RNAII-*oriV* hybrid, RNAI must bind to the

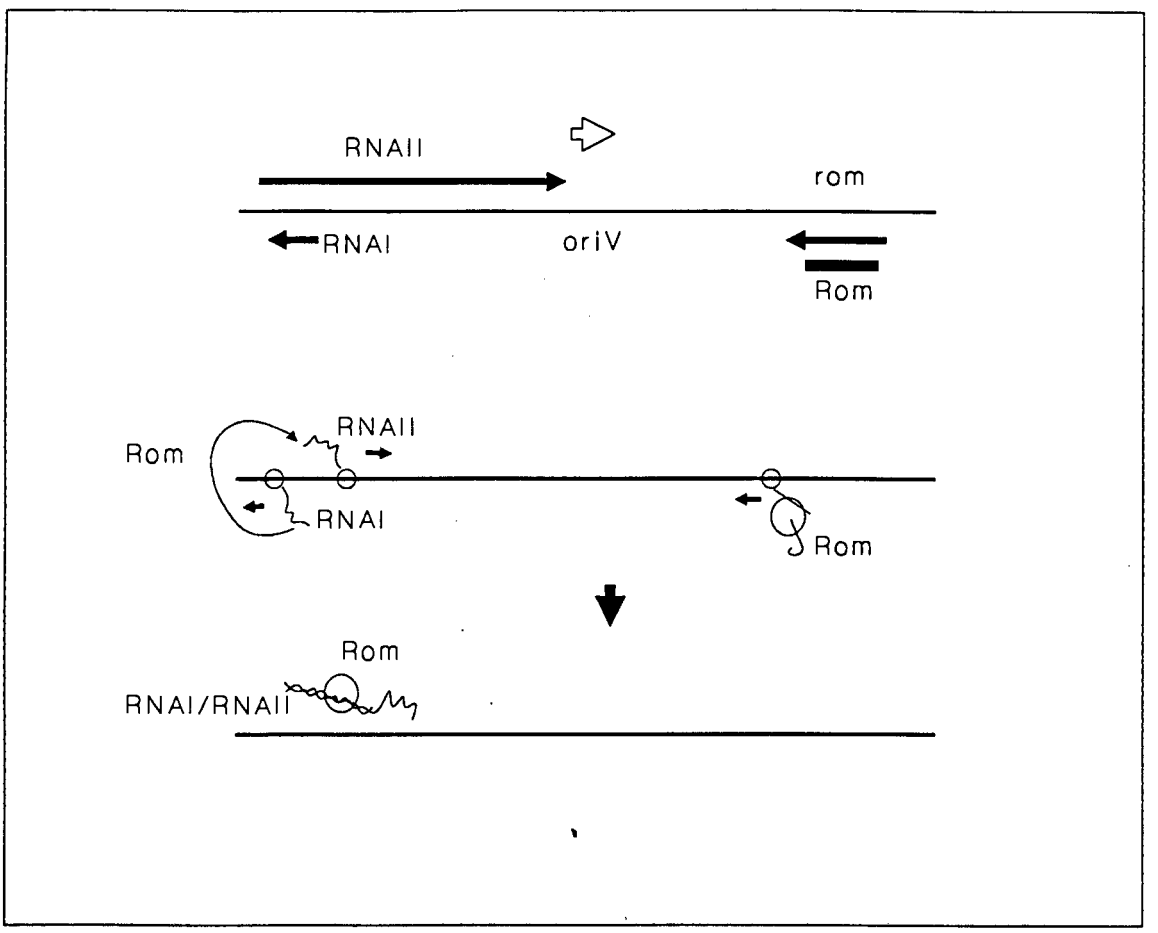


Fig. 1.4. Replication control in ColE1 plasmid. The 108 nucleotide AS RNAI is transcribed from the opposite strand to the RNAII preprimer. RNAI hybridizes with RNAII preventing it from interacting with the *oriV* locus. RNAI-RNAII hybrid formation is stabilized by Rom protein. This AS control mechanism determines the efficiency of primer formation and the subsequent initiation of the leading strand synthesis in the replication of ColE1 plasmid (after Masukata et al., 1987).

nascent RNAII transcript Tomizawa, 1986). Recent studies suggest that RNAII (consisting of 241 nucleotides) adopts a secondary structure with similar conformation to the RNAI folded structure, therefore favoring an efficient interaction between RNAI and RNAII (Tomizawa, 1990a). Both, the structural conformation and the rate of interaction between the two RNA species (RNAI and RNAII) appear to play a significant role in this regulatory mechanism. The RNAI-RNAII interaction process can be summarized in three consecutive stages. The complementary sequences and conformations present in both RNA species (RNAI and RNAII) allow them to form reversible base pair associations termed initial "kissing". A stable interaction between the single stranded 5' RNAI end (or "tail") and the complementary loopIV (or anti-tail) in RNAII follows, while double-stranded sections of the two molecules are formed. The spread of these interactions results in the formation of a duplex structure along the whole extension of the two RNA molecules.

Mutagenesis experiments and structural analyses indicate that the presence of fine structure elements such as a "tail" and loop to loop interactions appear to be essential for stabilizing the hybrid formation (Tomizawa, 1990a; Polisky et al., 1990).

ColE1 replication is gradually inhibited when there is an increase in the plasmid copy number. The plasmid copy number in turn, has been shown to be proportional to the concentration of RNAI. A decrease in the plasmid copy number is followed by a fall in the concentration of RNAI.

A DNA region situated downstream of the *oriV* locus was shown to be involved in the additional control of the plasmid copy number due to the presence of a *rom* or *rop* gene encoded dimeric protein, which facilitates the interaction between RNAI and RNAII (Cesareni et al., 1982; Tomizawa and Som, 1984). The molecular mechanism of Rom function has been studied in detail (Eguchi and

Tomizawa, 1990). Rom protein has been reported to bind the RNAI-RNAII complex. This protein recognizes the general stem-loop structure rather than its sequence, and protects the stems of both RNAI and RNAII from nuclease digestion (Fig. 1.4) (Eguchi and Tomizawa, 1990). Rom protein stabilizes the RNAI-RNAII complex decreasing its dissociation constant (Tomizawa, 1990b). Subsequently, Rom catalyzes the conversion of an unstable intermediate complex into a more stable one, which results in the formation of the RNA hybrid (Tomizawa, 1990b).

1.4.1.2. Transcriptional termination

1.4.1.2.1. IncFII plasmids. The replication of these *E. coli* plasmids relies on the *repA* gene product (RepA) as well as on host encoded proteins. The copy number varies between 1 or 2 per chromosome, and it is basically controlled by the regulation of RepA protein expression (reviewed by Womble and Rownd, 1988).

Plasmid R1 belongs to the IncFII group. Its replication frequency has been shown to be tightly controlled by the rate-limiting protein RepA, which promotes replication by interacting at the origin of replication, *oriV*. It has been reported that the presence of a DNA sequence, CIS, situated between *repA* and *oriV*, stimulates a cis favored RepA type of interaction (Masai and Arai, 1988).

Two repressors are involved in the control of *repA*transcription. A *copB* encoded repressor protein, which binds to an operator site located within one of the two *repA* promoters, rendering *repA* transcription partially repressed due to residual low level constitutive transcription from the second *copB* promoter (Riise and Molin, 1986).

The *copA* encoded AS RNA of 91 nucleotides, is transcribed in the opposite direction to the transcription of the *repA* gene. It acts as a repressor by forming a hybrid with the CopT target sequence located in the leader region of the *repA* mRNA (Fig. 1.5) (Womble et al., 1984). Evidence has shown the presence of a small open reading frame (*tap*) preceding the *repA* gene. The *repA* start codon is overlapped by the *tap* stop codon (Blomberg et al., 1992). Translation of *repA* depends on *tap* translation not on the *tap* gene product. *copA* AS is complementary to the upstream sequence of the *tap* ribosome binding site. *copA* inhibits translation of the *tap* leader peptide, impeding *repA* translation as a consequence (Blomberg et al., 1992). Loop structure formation by both CopA and CopT has been demonstrated and analyzed by sequencing and structure specific RNAse degradation (Wagner and Nordström, 1986). Mutations affecting copy number and mapping in the region distributed along the CopA/CopT Stem-loopII have established this section as the most critical structure mediating interaction between the respective molecules. Thus, only a few base pairs have been shown to participate in the initial "kissing", or CopA-CopT interaction (Wagner et al., 1988).

Kinetic studies performed by using reaction substrates which blocked the stages after the formation of the initial CopA-CopT complex served to estimate the strength of the CopA-CopT interaction. Further analysis suggested the interaction of 7 or 8 nucleotides from each component in the initial CopA-CopT complex (Persson et al., 1990a).

Other elements appear to participate in the CopA regulatory system. Two small genes (coding for polypeptides of 7 and 3kD) are situated in the *repA* mRNA leader region. Inhibition by CopA RNA may be influenced by the translation of these two genes. It has been suggested that this effect on CopA inhibition is due

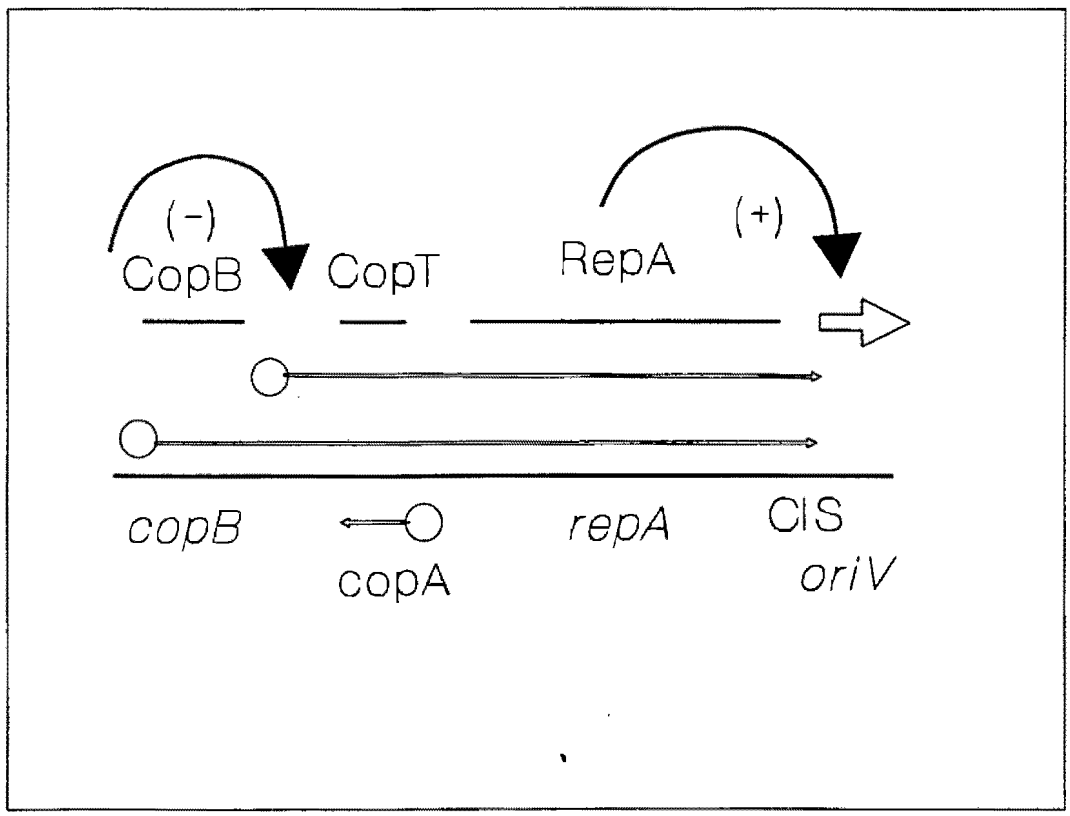


Fig. 1.5. IncFII plasmid replication control system. The 91 nucleotides *copA* AS RNA is transcribed in the opposite orientation to the *repA* gene. RepA protein promotes replication of the plasmid by interacting with the *oriV* locus. *copA* AS RNA impedes *repA* translation by forming a hybrid with the CopT target sequence located in the leader region of the *repA* mRNA. The *copB* gene encodes the CopB repressor which inhibits transcription of *repA* gene (after Riise and Molin, 1986).

to ribosome availability in the cell and would influence plasmid copy number (Wagner et al., 1987). Moreover, a transcriptional pause site has been located upstream of the initiation of the *repA* coding region. It has been proposed that this pause site could retard transcription to enable the mRNA structure to become more sensitive to CopA than the proper full-length mRNA structure (Dong et al., 1987). RNaseIII has been implicated in the processing of *repA* mRNA once a CopA-CopT complex has formed. Thus, cleavage of the CopA-CopT complex by RNaseIII would serve to maintain a limiting amount of active *rep* mRNA. This has been shown by both, *in vivo* and *in vitro* studies (Blomberg et al., 1990).

IncFII plasmids represent an AS RNA control system that may be influenced by a number of factors. Plasmid constructs were developed by replacing the *repA* promoter sequence by a thermoinducible lambda PL promoter (Larsen et al., 1984). The induced transcription from PL at high temperature caused inhibition of CopA production. As a consequence, a complete lack of regulation by CopA led to increased levels of repA mRNA, and also to simultaneous reduction in the inhibition of translation.

1.4.1.2.2. **pT181 and related plasmids.** Comprehensive studies have been carried out on the control of replication of the *Staphylococcus aureus* plasmids pT181 and pC194 (Alonso and Taylor, 1987). In pT181, *cop* and RNAI are AS RNAs complementary to the leader region of the *repA* mRNA. Involvement of *cop* and RNAI AS RNA molecules (Kumar and Novick, 1985) in premature transcription termination or attenuation (Highlander and Novick, 1990) of the *repA* translation has been reported. It has been proposed that in this case the AS RNA-mRNA interaction induces the formation of a secondary structure that resembles a Rho-independent transcriptional terminator upstream of the *repA* gene.

1.4.1.3. Uncertain mechanism: plasmid R6K

This plasmid is characterized by the presence of three potential origins of replication α,β and γ. Only α and β play a major active role *in vivo* (Crosa, 1980). *ori*γ appears to be frequently inactive. Two transcripts are encoded by the *ori*γ region: a silencer AS RNA, and an activator RNA involved in the *ori*γ function. The silencer AS RNA is transcribed from a promoter located at the side of the *ori*γ activation region. This silencer AS RNA appears to repress *ori*γ by binding to the activator RNA (Patel and Bastia, 1987). A replication initiator π protein may play a role in stabilizing the AS RNA-mRNA hybrid, in a manner similar to the ColE1 Rom protein. It has been demonstrated that π protein promotes the activator/silencer hybrid formation *in vitro*.

1.4.2. Control of Conjugation

1.4.2.1. Transcriptional termination: IncF plasmids. The control of the expression of conjugative transfer functions by AS RNA is different from that utilized in plasmid replication. After the transfer of plasmids between bacteria has occurred, F-like conjugation systems continue to be active for a relatively long time and involves a few generations. Two genes, *fin*P and *fin*O, are responsible for the control of the conjugative transfer of the F-like sex factors in *E. coli* (Ippen-Ihler and Minkley, 1986). The *fin*P gene is located within the F plasmid and it is transcribed into a FinP AS RNA product of 105 nucleotides (Dempsey, 1987). The FinP RNA target *fis*O, is located within the *tra*J mRNA, which encodes a positive activator of transcription of the *tra* operon. It had been postulated that the FinP-*fis*O interaction determines transcription termination, and as a consequence the synthesis of a shorter *tra*J mRNA

transcript (Dempsey, 1987). Recently, it has been established that the participation of a functional *fin*O gene is also required in this system. The *fin*O RNA acts by stimulating FinP AS RNA activity. First, FinP RNA is stabilized by *fin*O RNA. In addition, FinP RNA interaction with *tra*J mRNA appears to be catalized by *fin*O RNA. A model describing the FinOP action has been proposed by Frost et al. (1989). This model was supported by evidence from mutagenesis experiments performed in both *fin*P and *fin*O RNA molecules. The interesting and unique aspect of this system is that one RNA molecule stimulates the AS RNA activity on a third RNA molecule.

Inactivation of *fin*O by insertional mutagenesis with IS3 derepresses conjugative transfer of the F plasmid (Cheah and Skurray, 1986). *fin*O deficiency can be complemented by the presence of certain F-like plasmids. This complementation restores the Fin+ phenotype which represents inhibition of fertility.

Transcription from the *fin*P promoter is rather weak and gives rise to low levels of FinP RNA, which eventually accumulates over a long time to reach critical concentrations. This could explain the fact that after transfer to plasmid-free bacteria the naturally repressed F-like transfer system continues to be derepressed for some generations.

Conjugative experiments involving high copy number DNA fragments carrying the *tra*J promoter, its leader sequence, but in which the *fin*P promoter was absent, produced at least a 100-fold derepression of R100 transfer (Dempsey, 1989a). Transcription of the *tra*M gene, located upstream of *tra*J, generates transcripts that overlap the *tra*J open reading frame. High concentrations of these transcripts can titrate FinP AS RNA. Furthermore, transcription of *tra*M, which is activated by the positive regulator TraJ, could magnify the derepression of *tra*J by sequestering FinP RNA (Dempsey, 1989b).

1.4.3. Control of Host-killing genes

1.4.3.1. Ribosome binding: R1 plasmid. The *par*B locus of plasmid R1 is composed of three genes, *mok*, *hok*, and *sok*, which are involved in plasmid stability by "postsegregational killing" (Gerdes et al., 1986b). During cell division, cells which lose their plasmids are eliminated by a mechanism dependent on the *parB* locus. While cells still contain their plasmids, transcription of *sok* produces an AS RNA which interacts with *hok* mRNA forming a *hok* mRNA-*sok* RNA hybrid. This hybrid is then cleaved by RNase III. Therefore, *sok* AS RNA inhibits *hok* mRNA translation and thus the synthesis of the lethal product. While the plasmid is still present in the cells the AS RNA is highly expressed, but unstable. In these cells a stable *hok* mRNA is also produced at low levels. After cell division, in cells where the plasmid DNA template is lost, *sok* RNA levels decrease due to its instability. In contrast, *hok* mRNA levels remain relatively constant due to its stability. The end result is that *sok RNA* levels are not sufficient to inhibit *hok* mRNA expression. Therefore, translation of *hok* mRNA into its lethal product occurs, causing cell death (Fig. 1.6) (Gerdes et al., 1988).

Another component of this locus has been denoted the *mok* (mediation of killing) gene, which is situated in the region upstream of the *hok* gene and overlaps the *hok* open reading frame. Both, *mok* and *hok* are transcribed in a single mRNA. Transcription of *mok* mRNA precedes *hok* mRNA in this single transcript (Gerdes et al., 1990a). It has been proposed that *mok* is translationally coupled to *hok*. Mutagenesis of *mok* causing translational termination in a site previous the overlap with *hok*, disturbed the expression of *hok*.

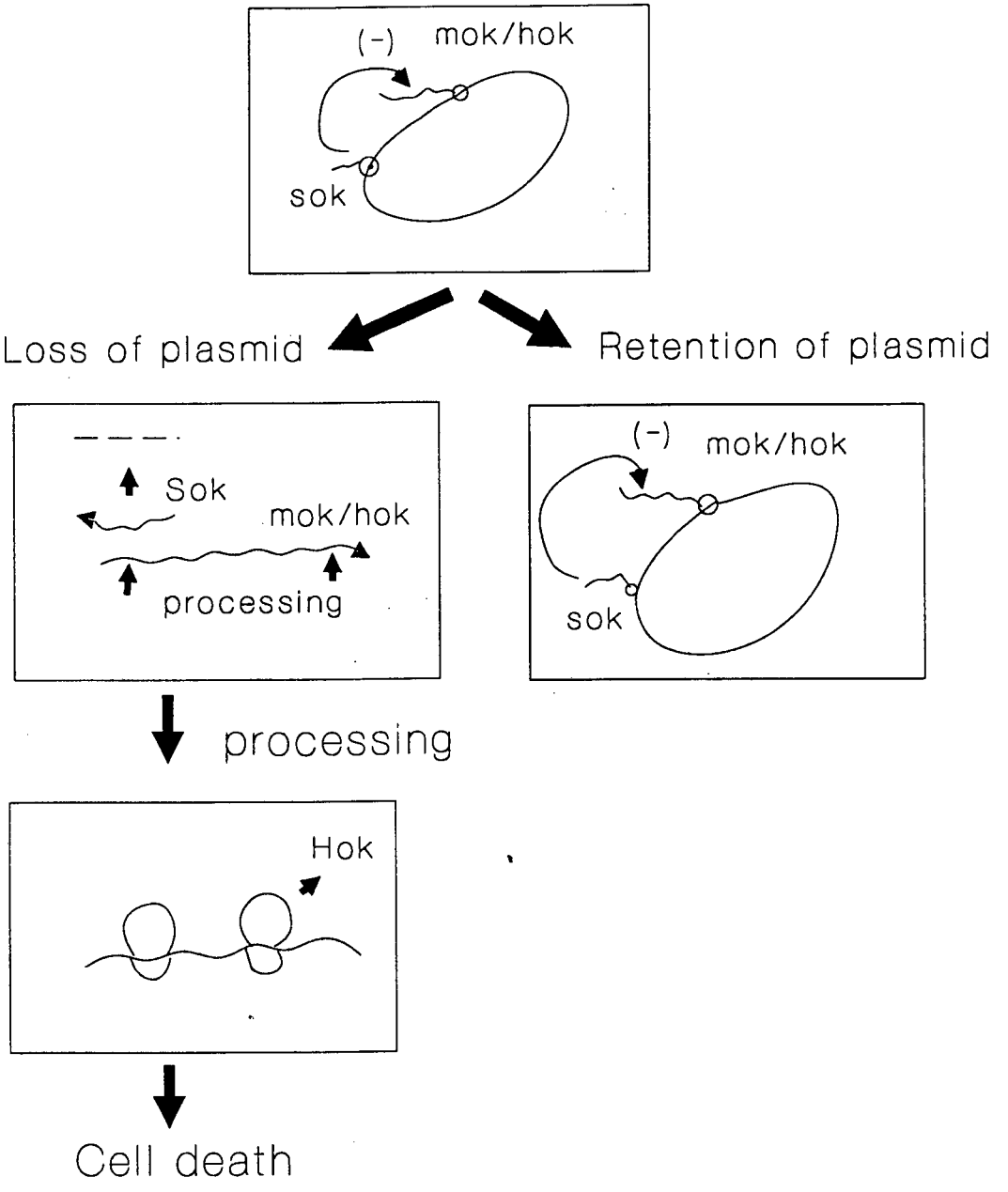


Fig. 1.6. Killing of plasmid free segregant bacteria by the parB region of R1. *sok* AS RNA interacts with *mok/hok* mRNA (both genes encoded by a single mRNA) and inhibits translation of *hok* mRNA into the lethal product. After cell division in cells where the plasmid is lost, *sok* RNA levels decrease and are not sufficient to inhibit *hok* mRNA translation. The *hok* mRNA lethal protein is produced causing cell death. In cells where the plasmid is still present the synthesis of the lethal product is inhibited by the *sok* AS RNA (after Gerdes et al., 1988).

1.4.4. Control of transposition

1.4.4.1. Ribosome binding: IS*10* transposon. An AS regulatory mechanism is responsible for the translational control of the transposase gene (*tnp*). This gene is located within the insertion sequence IS*10* of the tetracycline transposon Tn10. The pOUT AS RNA of 69 nucleotides represses the transposase pIN mRNA by the formation of a hybrid, which restricts access to the ribosome-binding site (Ma and Simons, 1990). RNA-OUT and R1 CopA AS RNA share many structural properties (Kittle et al., 1989). However, as a system, the AS RNA-OUT presents essential differences from copy number AS RNAs.

Primarily, RNA-OUT activity depends on its highly stable structure with a half life of 60 min. Mutagenesis experiments caused a reduced stability of the RNA-OUT stem structure. The decrease in the stability of the RNA-OUT structure cancelled the AS RNA repressor activity. This condition was restored when a new set of mutations enabled the formation of the original RNA-OUT stem structure. The stability of this AS RNA is somehow due to its structure that is not vulnerable to the action of ribonucleases, such as RNaseIII. Mutagenesis can make the AS RNA structure sensitive to degradation by RNase III. Cleavage by RNaseIII produced an irreversible inhibition of the transposase translation in experiments in which a mismatch mutation is produced at position 15 in the RNA-OUT stem structure (Case et al., 1989).

Another difference from plasmid copy number AS control systems is represented by the fact that transcription of RNA-OUT occurs from a weak promoter (Case et al., 1988). Due to its high stability and low transcription levels, the concentration of RNA-OUT is not directly responsive to variations in the transposon copy number. Therefore, this mechanism appears to control the accumulation of IS*10* by a rate limiting interaction between RNA-OUT and RNA-IN.

1.4.5. Control in bacteriophages

1.4.5.1. Transcript stability: lambda phage. AS RNA regulatory systems have been demonstrated in both *E. coli* phage lambda and the related *Salmonella* phage P22. Two main functions are common to the early positive transcription factors, cII in lambda, and c1 in P22. The first, is to induce transcription of genes involved in lysogeny, and the second, is to induce a delay of late gene expression. In the second function, an AS RNA of 220 nucleotides appears to be involved. CII induces transcription of this RNA from the PaQ promoter. The AS RNA is transcribed in the opposite orientation within the late gene Q and acts by repressing Q gene expression (Hoopes and McClure, 1985). CII expression itself is also controlled by an AS RNA. Since CII is involved in establishing lysogeny, the repression of CII by an AS RNA promotes the lytic pathway. The OOP AS RNA of 77 nucleotides is complementary to the 3' end of the cII gene (55 nucleotides) and to a 22 nucleotide intercistronic sequence between genes cII and O. OOP RNA regulates the expression of cII by binding to the cII-O dicistronic mRNA. The hybrid is then cleaved by RNaseIII, inactivating cII mRNA (Krinke and Wulf, 1987). Experiments performed *in vivo* and *in vitro* have demonstrated that RNaseIII cuts at a position 13 nucleotides from the 3'end of the RNA hybrid (Krinke and Wulf, 1990).

1.4.5.2. Ribosome binding: Phages P22, P1, and P7. Binding of phage repressors to phage P22 DNA is affected by Ant, an antirepressor protein. Ant expression is controlled by Sar AS RNA. The interaction between the Sar RNA of 71 nucleotides, and the *ant* mRNA occurs via a single stranded RNA loop, which contains a sequence complementary to the ribosome binding site. A RNA hybrid structure is rapidly formed (Liao et al., 1987). Kissing between the two RNA molecules has been suggested to be enough to repress translation by impeding interaction with the ribosomes. The Sar RNA acts by inhibiting Ant synthesis

after the Ant protein has initially been overexpressed, so the infection may proceed to the lysogeny stage. Experiments in which mutations were introduced into the Sar RNA gave rise to clear plaques demonstrating the inability to establish lysogeny.

It is of interest to analyze how the inhibition by Sar and Ant expression are coordinated. Initial transcription of *ant* has an effect on *sar* transcription. Another regulator, Arc, is also acting in the system. This protein represses later transcription of *ant*. Transcription of *ant* is diminished by the Arc repressor while *sar* is still being transcribed. Therefore, two regulatory mechanisms have a coordinated action at the levels of transcription and translation. *ant* transcription is down regulated by Arc at the same time *ant* mRNA translation is inhibited by Sar RNA (Wu et al., 1987).

The presence of an AS RNA linked to its target mRNA has been elucidated in two closely related bacteriophages, P1 and P7 (Citron and Shuster, 1990). c4 AS RNA modulates the expression of the *ant* gene. *ant* encodes an antirepressor protein that must be repressed in a lysogen. Three genes are present in this region, c4, *orf*X, and *ant*. They are transcribed in the same order from a promoter located upstream of c4. *orf*X is a short open reading frame with unknown function which overlaps the *ant* gene start. c4 has a complementary sequence to the ribosome binding site located upstream the *orf*X gene. Translation of *orf*X appears to be required for the *ant* gene to be expressed. c4 RNA has been postulated to be acting as an AS RNA. It is not translated into a protein product. Mutagenesis of c4 abolished its role as an inhibitor of *ant* expression. In addition, c4 has been proposed to interact with the ribosome binding sequence of the *orf*X mRNA. This interaction inhibits both, *orf*X mRNA and *ant* mRNA translation. However, the mechanism by which this c4 RNA controls *ant* expression remains to be clarified.

1.4.5.3. Transcriptional processing: phage ϕH. AS RNA has been shown to participate in the developmental control of the phage ϕH of *Halobacterium salinarium*, an extremely halophilic archaebacterium. This phage is able to grow lytically as well as to establish lysogeny as a stable plasmid in *H. salinarium* (Zillig et al., 1988). When ϕH grows lytically, T1 is the first transcript produced. The function of the T1 protein product has not been elucidated. However, regulation of T1 expression seems to be critical for phage growth. T1 production has been shown to be regulated through an AS RNA transcript, T_{ant}, of 151 nucleotides. T_{ant} binds to the T1 Shine-Dalgarno sequence inhibiting interaction with ribosomes, and processing of the RNA duplex has been demonstrated. The presence of an AS RNA control mechanism in archaebacteria indicates that AS RNA regulation evolved at a time before eukaryotes, eubacteria and archaebacteria diverged during evolution (Stolt and Zillig, 1993).

1.4.6. Regulation of bacterial genes

1.4.6.1. Ribosome binding

1.4.5.1.1. OmpF synthesis in *E. coli*. A few examples of chromosomal AS RNA systems have been identified, thus expanding the spectrum in which this regulatory mechanism appears to control gene expression.

The *E. coli ompB* locus is composed of two genes encoding the sensor-regulator proteins, EnvZ and OmpR. The expression of the *E. coli* outer membrane porins OmpC and OmpF is regulated by the concerted action of EnvZ (sensor) and OmpR (regulator) in response to osmolarity changes in the growth medium. Increased OmpF levels compared to OmpC occur under low osmolarity and the opposite occurs under high osmolarity conditions (Reviewed by Forst and

Inouye, 1988). The action of an AS RNA regulator is involved in this system. The *micF* AS RNA locus positioned at 47 min on the *E. coli* chromosome modulates the expression of the distant *ompF* gene located at 21 minutes on the *E. coli* chromosome (Mizuno et al., 1984). This was the first example of a chromosomally encoded AS RNA, and demonstrated that an AS RNA is not necessarily transcribed from the complementary DNA strand of the target gene to be able to produce an inhibitory type of interaction. *micF* RNA has been shown to act by inhibiting the expression of OmpF porin protein in response to a number of environmental signals, including osmolarity, temperature and ethanol induced stress (Misra and Reeves, 1987). The inhibitory effect of micF RNA has been implicated in the synthesis of low levels of OmpF and a decrease in *ompF* mRNA levels as a consequence of these external signals. Experiments using strains with or without a functional *micF*, demonstrated the inhibitory role of this AS RNA (Aiba et al., 1987). This was accomplished by analyzing the *de novo* synthesis of OmpF after an osmotic shift. The expression of this AS RNA appears to be controlled by OmpR (Fig. 1.7) (Coyer et al., 1990). *micF* RNA of 93 nucleotides, is complementary to the ribosome binding region and the 5'end of the *ompF* mRNA, including the initiation codon. *micF* RNA inhibiton of OmpF mRNA translation is considered to be a direct effect followed by a rate-limiting ribonuclease degradation of *ompF* mRNA (Andersen et al., 1989).

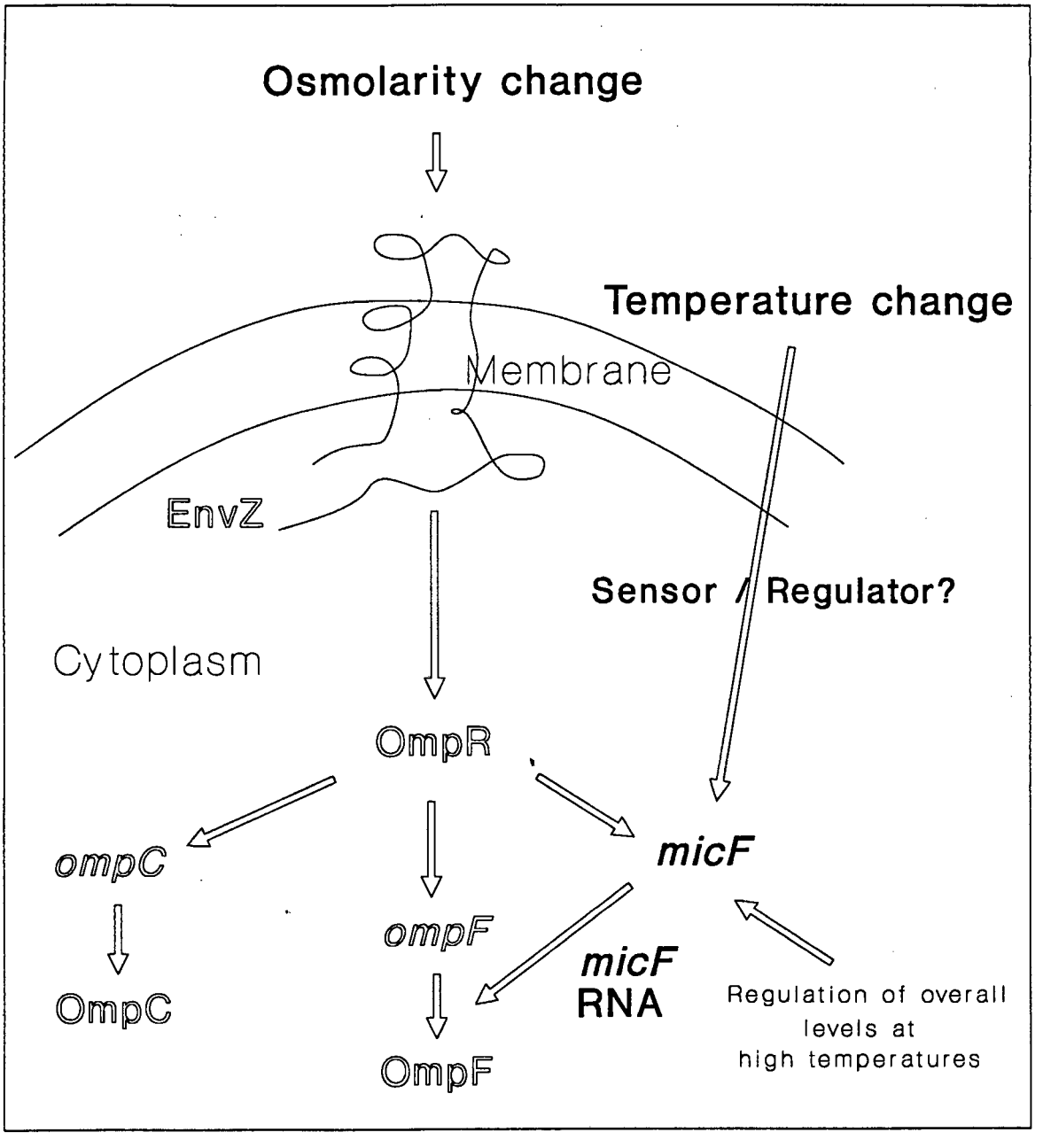


Fig. 1.7. Thermal and osmolal regulation of *micF* RNA and the porin proteins OmpC and OmpF. The *ompB* locus encodes for the EnvZ and OmpR proteins. *micF* AS RNA inhibits the synthesis of OmpF in response to a number of environmental signals. This model of regulation of *micF* suggests that the outer membrane porin protein OmpR plays a role in osmoregulation of *micF* RNA. However, other unknown factors independent of the *ompB* locus are involved in thermoregulation (after Coyer et al., 1990).

1.4.6.1.2. FtsZ synthesis in *E. coli*. Transcription from an inducible promoter situated within a long non-coding intercistronic region from the *E. coli dic*B operon generates DicF AS RNA (Faubladier et al., 1990). The DicB protein is involved in the inhibition of cell division. Transcription of *dicF* has also been associated with the blocking of cell division, especially at high temperatures (Bouché and Bouché, 1989). The *ftsZ* gene product is required for cell division and is inactive at high temperatures. Translation of DicF RNA into a protein product has not been demonstrated. DicF RNA of 53 nucleotides has been found to result from both, RNaseIII and RNaseE processing. DicF RNA has a complementary sequence to the *ftsZ* mRNA leader region including the ribosome binding site and the 5'start coding region (Tétart and Bouchés, 1992). Interaction of DicF AS RNA with the *ftsZ* mRNA complementary region has been implicated in the inhibition of *ftsZ* expression. DicF AS RNA inhibition of *ftsZ* expression could explain the effect of DicF RNA in the inhibiton of cell division blocking at high temperatures. It has been shown that the inhibitory effect of DicF RNA is lost by overexpression of a cloned *ftsZ* gene.

1.4.6.1.3. *gnd* expression in *E. coli*. 6-P-gluconate dehydrogenase (6PGD; EC 1.1.1.44) gene expression has been analyzed in *E.coli* as a model system for the study of gene regulation relative to the cell growth rate. An internal DNA sequence within the *gnd* structural gene encodes a cis-acting AS RNA complementary to the ribosome binding site of the *gnd* mRNA. This conserved internal complementary sequence (ICS) has also been found within the *S. typhimurium gnd* gene. AS RNA regulation of *gnd* gene expression is accomplished by the interaction of the ICS AS RNA with the *gnd* mRNA Shine-Dalgarno sequence causing translational inhibition.

Regulation of *gnd* expression by ICS-AS RNA appears to be associated with cell growth rate. At a certain growth rate a specific concentration of ribosomes is available for mRNA translation in the cell. Therefore, competition between free ribosomes and ICS-AS RNA for the *gnd* mRNA ribosome binding site occurs. The concentration of available ribosomes that eventually initiate translation is suggested to be the growth rate limiting factor. Under slow growth conditions a low level of ribosomes is available so the ICS-AS RNA mechanism acts by repressing *gnd* expression. Conversely, during rapid growth the concentration of ribosomes increases and translation of *gnd* occurs (Carter-Muenchau and Wolf, 1989).

1.4.6.1.4. *fatA* expression in *Vibrio anguillarum*. In the pathogen *V. anguillarum* a 65kb virulence plasmid encodes the iron uptake system. The plasmid encoded region responsible for iron transport is composed of four linked genes, *fatD, fatC, fatB,* and *fatA. fatA* gene product is represented by an outer membrane protein. The synthesis of this protein appears to respond to iron levels.

RNA_{α} is a 650 nucleotide AS RNA transcribed in the opposite orientation from the *fatB* DNA region. AS RNA_{α} is complementary to the 5'leader region of the *fatA* mRNA. Expression of RNA_{α} results in a slight decrease in *fatA* mRNA levels and a subsequent major decrease in FatA synthesis. It is suggested that AS RNA_{α} acts by inhibiting *fatA* mRNA translation by interacting with the 5' leader region thus impeding ribosome binding. Under iron-rich conditions RNA_{α} is induced and *fatA* expression is switched off completely. Regulation of *fatA* expression by AS RNA_{α} has been postulated to act as a fine-tuning system. Evidence has shown the presence of an iron-responsive protein regulator which appears to be acting at the level of *fatA* transcription (Waldbeser et al., 1993).

1.4.6.2. Transcriptional termination: CRP synthesis in *E. coli*. The *E. coli crp* gene encodes the cAMP receptor protein, which is also a sequence-specific DNA binding protein (catabolite receptor protein, CRP). CRP has been demonstrated to be involved in transcriptional regulation of certain genes involved in a number of metabolic activities. This protein requires cAMP as an allosteric effector to exhibit its regulatory function. CRP-cAMP may act either as a positive (Pastan and Adhya, 1976) or a negative (Prusiner et al., 1972; Musso et al., 1977; Movva et al., 1981) regulator depending on the target system.

In vitro experiments showed that the divergent *tic* AS RNA is involved in a mechanism of autoregulation of *crp* gene expression in *E. coli*. *tic* RNA is transcribed in the opposite orientation, from a site 2bp upstream of the transcription initiation site of *crp* mRNA (Fig. 1.8) (Hanamura and Aiba, 1991). Expression of *tic* AS RNA is stimulated by binding of the CRP-cAMP complex to the *tic* promoter region. The first 10 nucleotides of *tic* RNA are complementary to the 5'end of the *crp* mRNA. Autoregulation is performed when the *tic* RNA interacts with the 5'end of the *crp* mRNA forming a terminator-like structure that causes transcription termination (Okamoto and Freundlich, 1986). In *in vivo* studies, inactivation of the *tic* RNA promoter also showed impaired regulation by cAMP (Okamoto et al., 1988). However, a comprehensive *in vitro* analysis of the molecular mechanism involved in the negative regulation of the *crp* gene, indicated that inhibition of *crp* transcription by CRP-cAMP could take place without the participation of *tic* RNA (Hanamura and Aiba, 1991). *In vitro* transcription studies on DNA fragments carrying the *crp* promoter region showed that *crp* transcription was specifically inhibited by CRP-cAMP. Furthermore, both *crp* and *tic* RNA promoters were shown to be differentially regulated by CRP-cAMP in a synchronized manner.

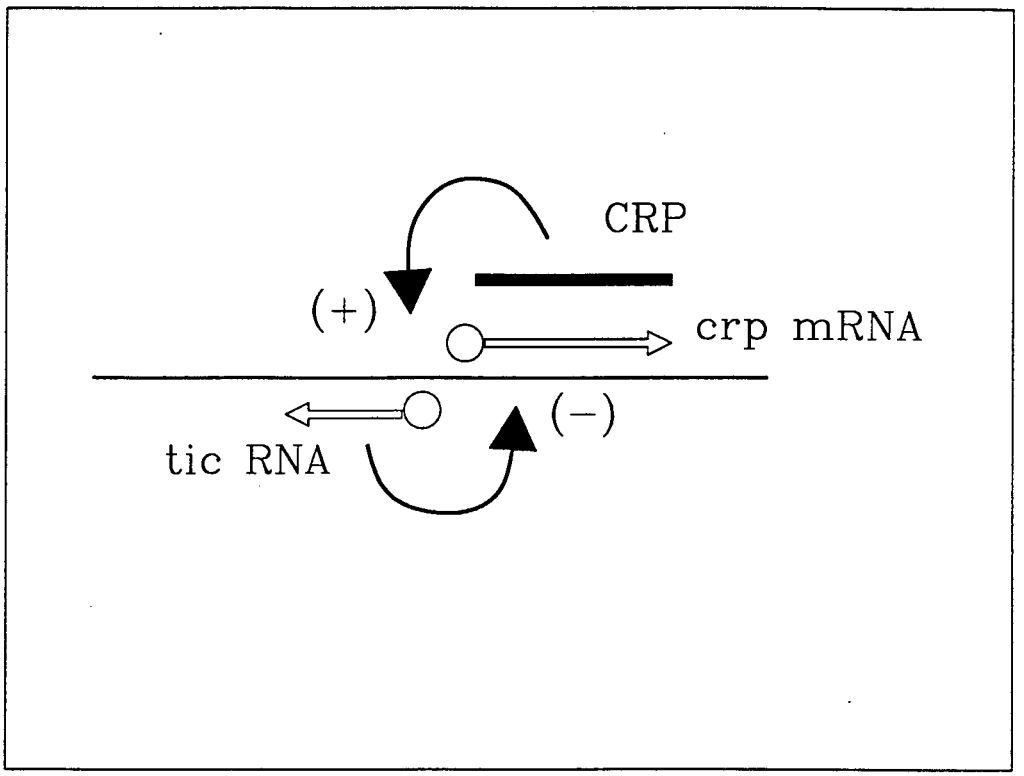
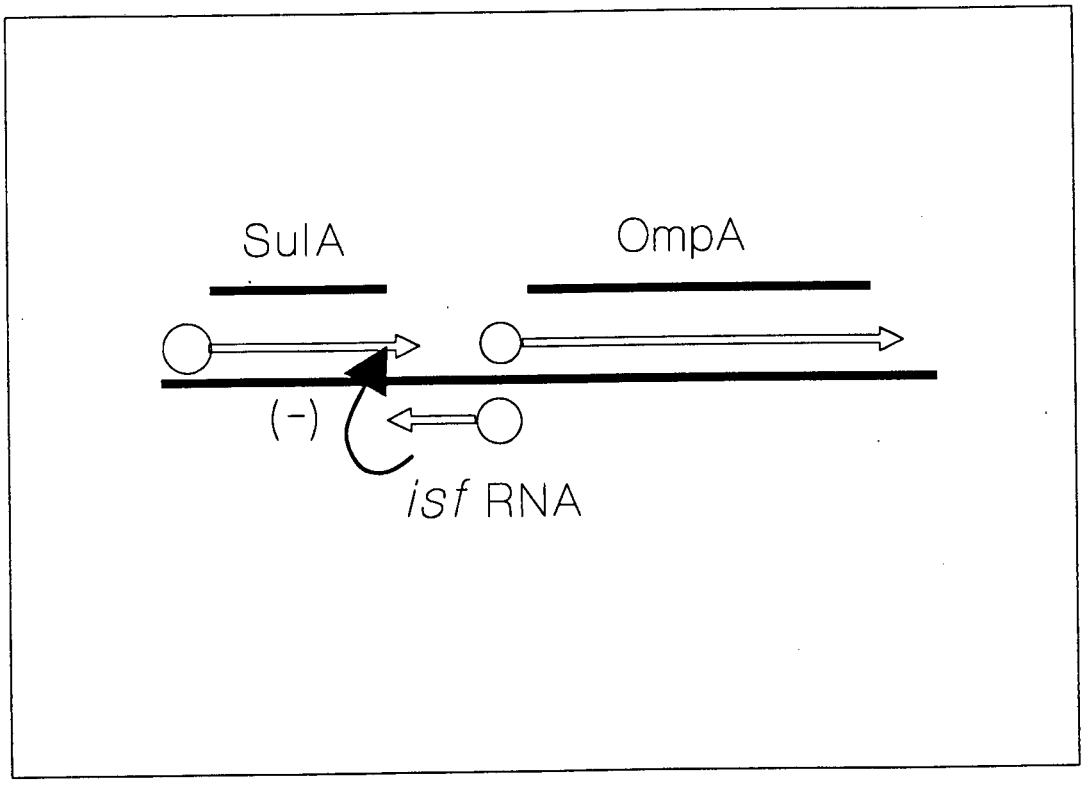


Fig. 1.8. tic RNA control mechanism. Transcription of the *tic* AS RNA in the opposite orientation to the *crp* mRNA is stimulated by the binding of the CRP-cAMP complex to the *tic* RNA promoter region. Autoregulation by *tic* RNA occurs when *tic* AS RNA interacts with the 5' end of the *crp* mRNA causing transcriptional termination (Okamoto et al., 1988).

1.4.6.3. Binding to the 3' mRNA region: SulA synthesis in *E. coli*. The 22 min region of *E. coli* chromosome is characterized by the presence of *sulA* and *ompA* linked genes. The *sulA* protein product appears to be involved in blocking cell division and is induced by a SOS cell response. *sulA* expression is normally repressed by LexA protein. The *ompA* gene encodes an outer membrane protein which occurs in large amounts and stabilizes the outer membrane. A further analysis of the *sulA-ompA* operon of *E. coli* revealed the presence of an untranslated *isf* RNA of 353 nucleotides. *isf* RNA was found to be transcribed in the opposite orientation from a promoter located in the region between *sulA* and *ompA* genes (Fig. 1.9). *isf* RNA is complementary to the 3' end of the *sulA* mRNA, and it has been proposed that it inhibits *sulA* expression during normal growth conditions. When *sulA* transcription is induced by the SOS response, the inhibitory effect of the *isf* RNA is suppressed leading to the significant expression of the *sulA* gene (Cole and Honore, 1989). Under SOS response conditions, a single transcript encoding *sulA* and *ompA* products is produced. Apparently this is due to transcription reading through the *sulA* terminator, the region between both genes and termination occurs at the *ompA* terminator.

Although all the examples described in this thesis involve natural AS RNA regulatory systems of prokaryote cells, this regulatory mechanism is also found to regulate various eukaryote genes.



1.9. *isf* RNA mechanism of control. The *sulA-ompA* operon of *E. coli* is characterized by the presence of *sulA, ompA* and the *isf* RNA coding region situated between the latter genes. Transcription of *isf* AS RNA of 353 nucleotides in the opposite orientation to the *sulA* mRNA inhibits expression of *sulA* mRNA into its protein product. The *sulA* protein appears to be involved in blocking of cell division (Cole and Honore, 1989).

1.5. Aims of this thesis.

This project represents a continuation of the studies on the regulation of the *C. acetobutylicum glnA* gene expression in both, the heterologous *E. coli* host and the original *C. acetobutylicum* host. The results obtained by Janssen et al. (1988, 1989) provided a detailed molecular characterization of the DNA sequence and the regulatory region of the *C. acetobutylicum glnA* gene. This work identified the presence of a putative AS RNA coding sequence and it was important to study the involvement of this sequence in the regulation of the *C. acetobutylicum glnA* gene in both the heterologous host, *E. coli* and the homologous host *C. acetobutylicum*. Since studies on the regulation of GS activity in *C. acetobutylicum* had not been carried out previously, basic physiological studies had to be done to establish the conditions for GS induction and repression. As it became apparent that other gene(s) are likely to be involved in the regulation of the *C. acetobutylicum glnA* gene, attempts were made to identify these other gene(s).

CHAPTER TWO

REGULATION OF *C. ACETOBUTYLICUM glnA* GENE EXPRESSION IN *E. COLI*

SUMMARY: The *C. acetobutylicum glnA* region is characterized by the *glnA* structural gene, the promoter sequences P_1 and P_2 situated in the region upstream of the *C. acetobutylicum glnA* gene and promoter P_3 located in the region downstream of *glnA*. Transcription from the promoter sequence P_3 in the opposite orientation of the *glnA* gene was demonstrated to express a 43-base AS RNA complementary to the 5'end of the *glnA* mRNA by RNase protection experiments performed in *E. coli* cells. The expression of GS activity or the *glnA* AS RNA were not regulated by N in the heterologous *E. coli* host, but the expression of the AS RNA in these cells was associated with decreased levels of GS activity. *C. acetobutylicum glnA* expression studies in a *E. coli* RNaseIII deficient mutant suggested the involvement of RNaseIII activity in the degradation of the *glnA* mRNA-*glnA* AS RNA hybrid.

2.1 INTRODUCTION

The DNA region encoding the *C. acetobutylicum* GS region was cloned by complementation of an *E.coli glnALG* deletion mutant. The GS was efficiently expressed in this *E. coli* mutant (Usdin et al., 1986). The structural *glnA* gene has been sequenced by Janssen et al. (1988). The entire open reading frame is represented by 1332 nucleotides which encode 444 aa acid residues. The *C. acetobutylicum* GS subunit has a predicted M_r of 49630, it is not adenylylated, and has a structure similar to the GSI structure found in other Gram-positive bacteria. When the GS aa sequence was compared with other GSI and GSII enzymes it contained the five conserved regions involved in the active site (Janssen et al., 1988). The derived aa sequence of the *B. subtilis* GS closely resembles that of *C. acetobutylicum* GS (Strauch et al., 1988).

C. acetobutylicum *glnA* gene expression in *E. coli* was found to be regulated by N levels from its own regulatory region (Usdin et al., 1986) under N-limiting conditions represented by GMM-15mM glu-0.15 mM gln and N-rich conditions, GMM-15mM glu-15mM gln. However, *E. coli* YMC10 cell growth was not optimal under these N conditions as glu is not considered to be a good N source (Reitzer and Magasanik, 1987). No *ntr* regulatory elements or $E\sigma^{54}$ consensus promoter sequences were found in the region upstream of the *glnA* structural gene (Janssen, 1988). The cloned *C. acetobutylicum glnA* DNA region of 6.5 kb present in the plasmid pHZ200 did not complement *ntrB* and *ntrC* *E. coli* mutants. As with the *B. subtilis glnA* region, the *C. acetobutylicum glnA* region did not appear to involve genes that form part of an enterobacteria-like global-N-regulatory system. The *glnA* gene was shown to be transcribed from two upstream promoter sequences, p_1 and p_2, in both *E. coli* and *C. acetobutylicum* cells. A palindromic sequence (37 bp) was present within the DNA region situated immediately upstream of p_1. Transcriptional initiation of the *C.*

acetobutylicum glnA gene in both *E. coli* and *C. acetobutylicum* was demonstrated by Janssen et al., (1990).

The DNA region situated downstream of the *glnA* structural gene was characterized by a set of inverted repeat sequences (158 bp in length). Transcription from this palindromic DNA region could give rise to a RNA molecule that is able to fold into different stem-loop structures. Experiments involving constructs in which this palindromic region was deleted from the *glnA* region significantly decreased the GS activity levels produced in *E. coli* cells. The existence of these inverted repeat DNA sequences has been implicated in transcriptional termination and mRNA stability (Janssen, 1990).

A third promoter sequence, p_3 directed in the opposite orientation to the transcription of *glnA* was found further downstream from the palindromic DNA region. p_3 was functional when subcloned into a promoter probe plasmid construct. In *C. acetobutylicum* and *E. coli* cells, transcription initiation from the p_3 promoter sequence confirmed the presence of a RNA transcript, which did not contain a ribosome binding site, a start codon or an open reading frame. This RNA sequence is complementary to the 5'end of the *glnA* region, including the ribosome binding sequence and appears to be a putative AS RNA (Fig. 2.1). A double mutation made in the -35 region of p_3 enhanced the promoter activity of this sequence and as a consequence lower levels of GS activity were produced in *E. coli* cells (Janssen et al., 1988; 1990).

This chapter deals with the regulation of *C. acetobutylicum glnA* gene expression in *E. coli*. Studies were carried out to elucidate the mechanism(s) affecting *glnA* gene expression, the N conditions and the gene(s) involved.

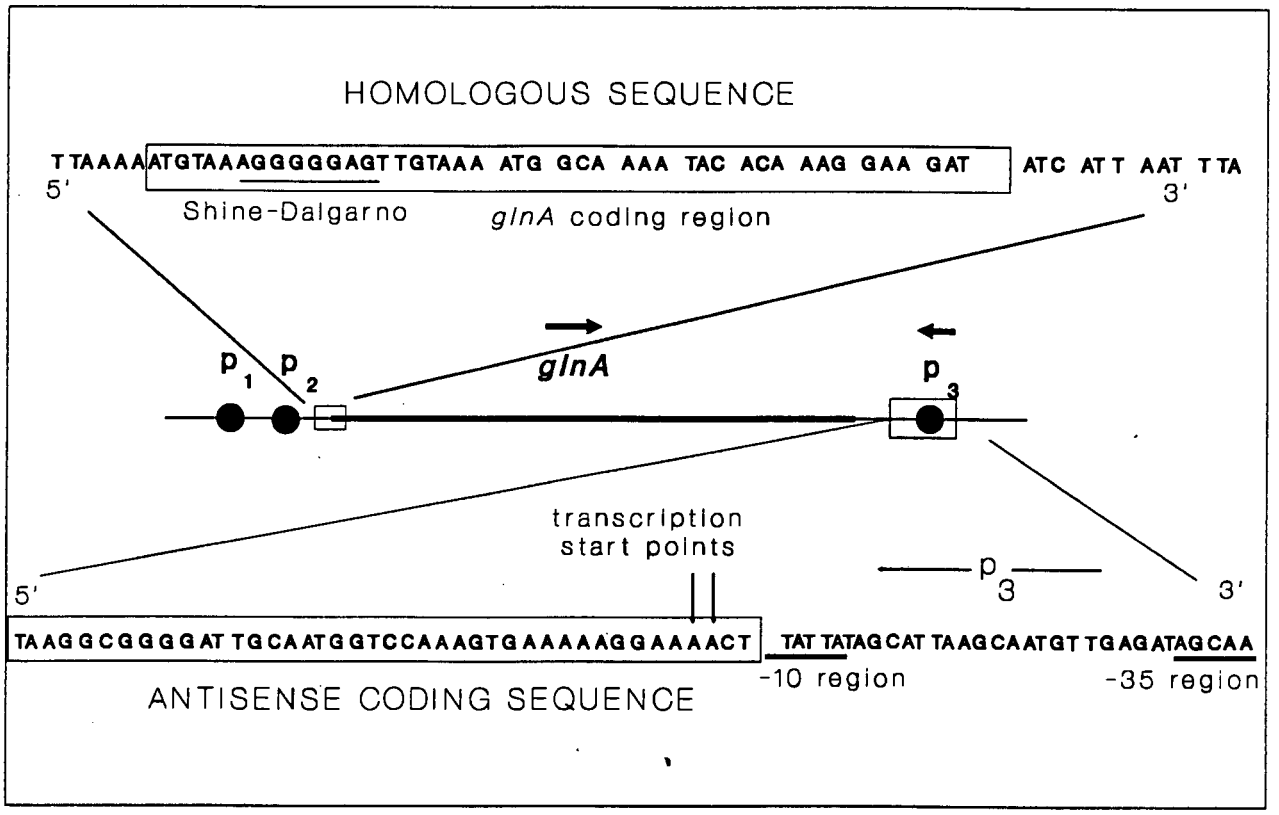


Fig. 2.1. (a) Diagram of the *C. acetobutylicum glnA* region cloned in pGln1300 (Fierro-Monti et al., 1992). Promoters (P_1, P_2, and P_3) are shown as large black dots; arrows indicate the direction of transcription; the AS RNA coding sequence and the homologous sequence are boxed.

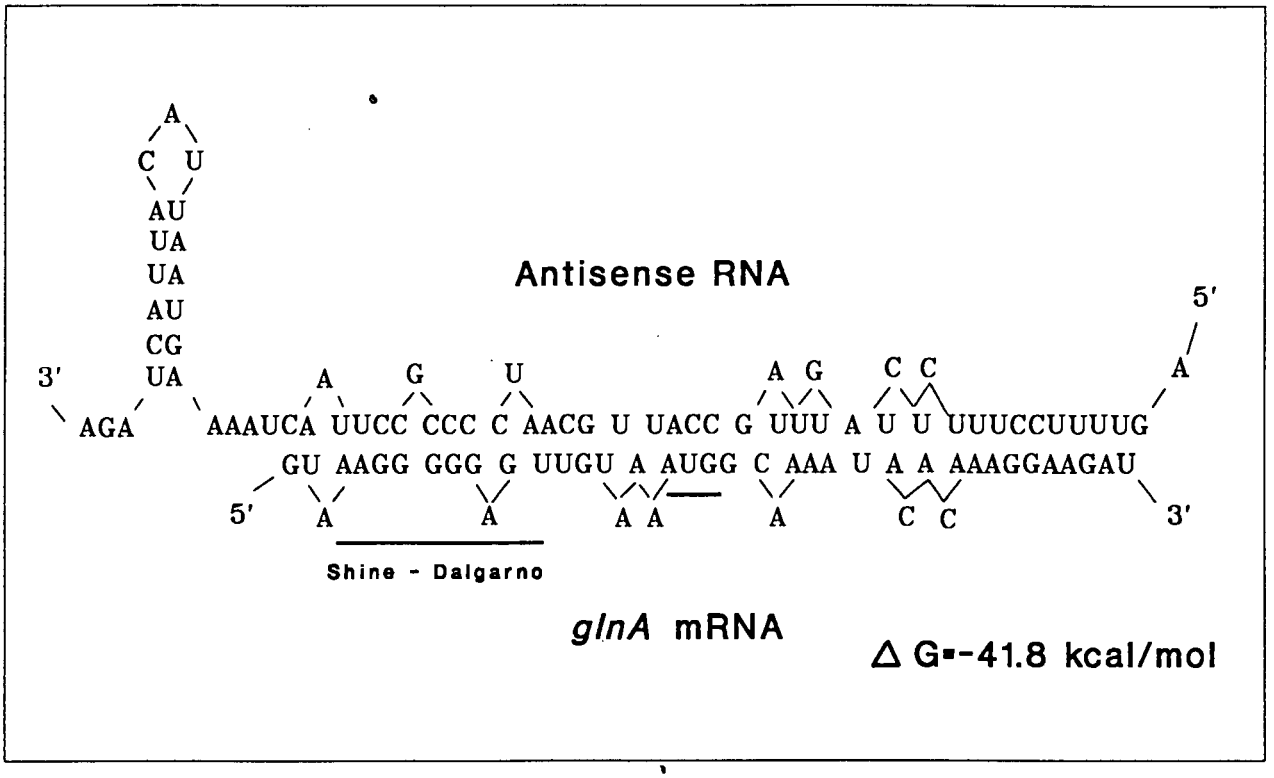


Fig. 2.1. (b) Proposed RNA-RNA hybrid formation between *glnA* mRNA and AS RNA (Janssen et al., 1990). 1kcal=4.184 kJ.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Appendix B.2. *E. coli* strains were grown in LB medium or M9 minimal medium (Miller, 1972) supplemented with the appropriate aa and antibiotics.

2.2.2 Preparation of nucleic acids. Plasmid DNA was prepared by the method of Ish-Horowicz and Burke (1981) (Appendix A.1.1-2). RNA from *E. coli* was extracted by the method of Aiba et al., (1981) and stored at -70^{o}C in 70% ethanol (Appendix A.1.12).

2.2.3 Plasmid constructions. Plasmid pHZ200, the primary DNA source (Fig. 2.2) (Appendix B.2) contained 6.5 kb of *C. acetobutylicum* DNA carrying the *glnA* structural gene, approximately 1,300 bp of upstream DNA sequence and 3,200 bp of downstream DNA sequence. This 6.5 kb *C. acetobutylicum* DNA fragment was cloned into the *Bgl*II site of pEcoR251 to yield pHZ200. Plasmid pHZ202 is an *Eco*RI deletion derivative of pHZ200 (Usdin et al., 1986). Plasmids pEcoR251 and derivatives pHZ200 and pHZ202, are similar high copy number plasmids. Plasmid pGln1300 (Janssen et al., 1990) contained the *C. acetobutylicum glnA* structural gene, approximately 1,300 bp of upstream sequence, and 614 bp of downstream sequence that included P_3 and the AS RNA coding region. This *C. acetobtuylicum Xba*I-*Bgl*II fragment was subcloned into the vector pLK34 (which is a high copy number plasmid) to yield pGln1300. pDR7 was constructed by deleting 480 bp from the downstream *glnA* region in pGln1300, including the AS RNA coding region and P_3. pTS-1 contained a *Sma*I-*Xba*I fragment from pGln1300 subcloned into the *Hin*dII site of plasmid pACYC177. pJP4 carried a

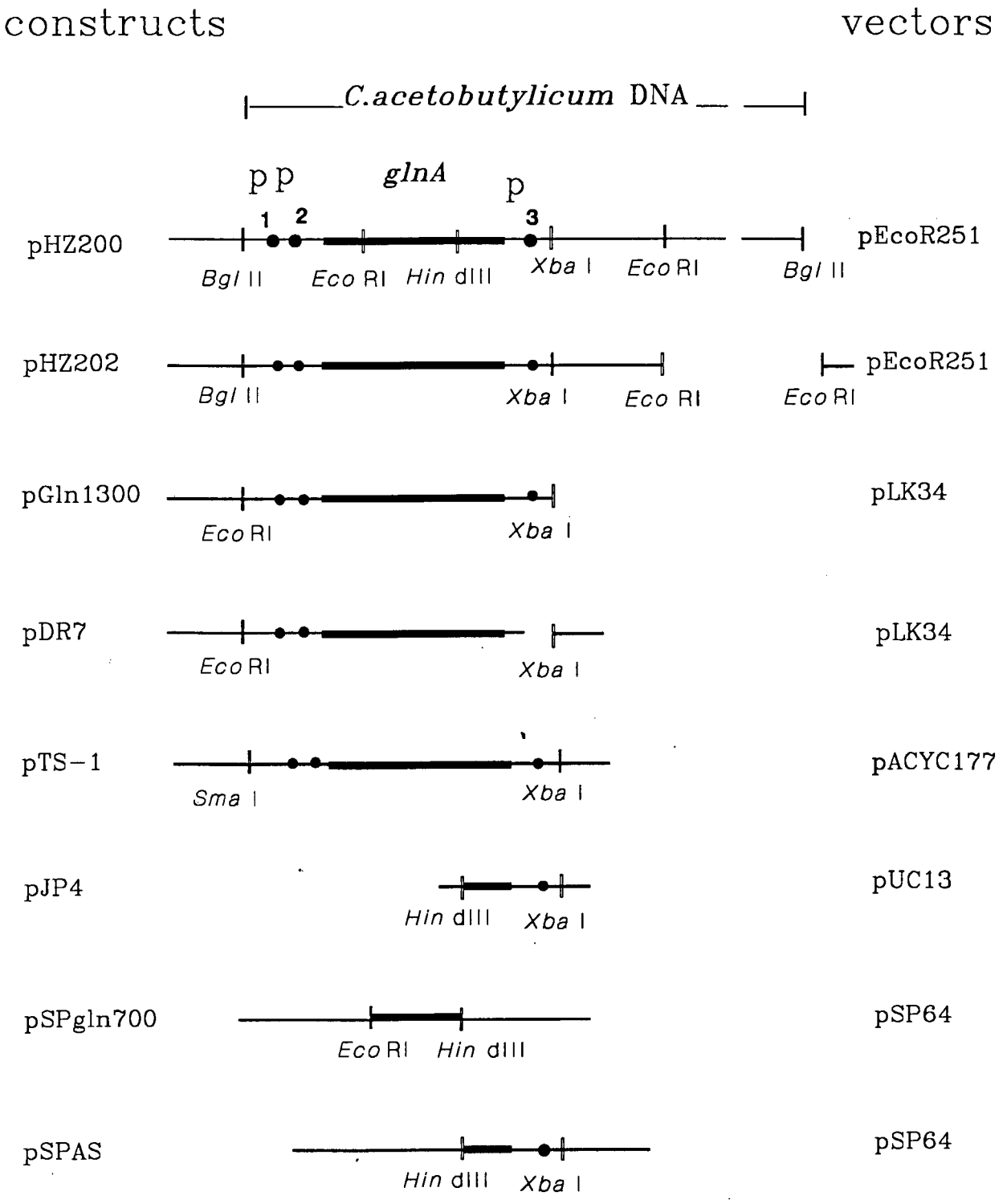


Fig. 2.2. Restriction map of the *C. acetobutylicum glnA* DNA region and the construction of various deletion plasmids.

728-bp *Xba*I-*Hin*dIII fragment from pGln1300, subcloned into the *Xba*I-*Hin*dIII sites of pUC13. This region included 111 bp of the 3'end of the *glnA* gene, the AS RNA coding region, and P_3. pSPgln700 contained an internal *Hin*dIII-*Eco*RI *glnA* fragment cloned into pSP64. pSPAS was obtained by subcloning the *Xba*I-*Hin*dIII fragment of pGln1300 into pSP64. Plasmids SPgln700 and pSPAS were used to generate single-stranded RNA probes for the RNase protection assay. Standard subcloning strategies and recombinant DNA techniques were used (Maniatis et al., 1984). Nucleotide sequences of plasmid constructs were confirmed by sequence analysis as described in Chapter 4.

2.2.4 RNase protection assay. The ribonuclease protection assay was used for the detection and quantitation of RNA species because of its high sensitivity compared to other procedures. A single-stranded radioactively labelled RNA probe complementary to the RNA species to be detected was utilized. This ribonucleoprobe was synthesized by *in vitro* transcription of an inserted DNA probe fragment from the SP6 bacteriophage promoter present in a SP6 transcription vector (Appendix A.1.13.2). The sample RNA and the ribonucleoprobe were mixed and incubated under suitable hybridization conditions (Appendix A.1.14.1). Ribonuclease degradation of the single-stranded ribonucleoprobe or other single stranded RNA present in this mixture left the ribonucleoprobe-target RNA duplex protected (Appendix A.1.14.2). This RNA duplex was separated on a polyacrylamide gel and visualized by autoradiography (Calzone, F. et al., 1987) (Appendix A.1.14.3). pSPgln700 and pASPAS were used as DNA templates for the synthesis of the internal *glnA* and the AS single stranded RNA probes, respectively. SP6 RNA polymerase was used to generate [α-^{32}P]UTP-labelled RNA probes by *in vitro* transcription, using the Maxiscript SP6 *in vitro* transcription kit supplied by Ambion, Inc., Austin, Texas. The procedure for the synthesis of high specific activity ribonucleoprobes,

the hybridization of the RNA probes with total RNA extracted from *E. coli* and *C. acetobutylicum,* and the RNase digestion of Hybridized RNAs are described in Apendix 1.

2.2.5 Enzyme assays. GS Activity was determined by the γ-glutamyl transferase assay according to Shapiro and Stadtman (1968) (Appendix 1.10). Total protein concentration was determined by the method of Bradford (1976) (Appendix 1.10.1). Cells were harvested by centrifugation and washed with 0.8% NaCl. Hexadecyltrimethylammonium bromide was used at a concentration of 1 mg/ml to inhibit growth and to render the cells permeable (Bender et al., 1977). Assays were performed at room temperature. GS specific activity were expressed as micromoles of g-glutamyl hydroxymate per milligram of protein per min. Percentages of repression/difference of GS activity were calculated by using the following formula:

$$\% \text{ Repression of GS} = 100 - [(\text{GS activity}_{\text{N-rich}} / \text{GS activity}_{\text{N-limiting}}) \times 100]$$

Repression ratios were calculated by dividing the enzyme activity detected in N-limiting cultures by that in N-rich cultures.

2.3 RESULTS AND DISCUSSION

2.3.1 Regulation of *E. coli* YMC10 *glnA* gene expression by N levels. The GS activity of the wild type *E. coli* YMC10 strain was examined under different N conditions in media which supported good cell growth. Regulation of the *E. coli* YMC10 GS activity by N levels was shown under N-rich or N-limiting conditions (Fig. 2.3). *E. coli* YMC10 was grown in M9 minimal medium supplemented with 15mM gln and 0.2% $(NH_4)_2SO_4$ for N-rich, and with 15mM gln for N-limiting conditions. The percentages of repression of GS activity varied between 70% and 86% when N-rich was compared with N-limiting conditions and GS activity repression ratios fluctuated between 3.3 and 7.3 depending on the sampling times. This control experiment was carried out to establish the N conditions that regulate the expression of the *E. coli* GS (Backman et al., 1981). It was important that the conditions for the study of the regulation of GS activity were also favorable for the growth of the cells. Growth of *E. coli* YMC10 was monitored in N-rich and N-limiting conditions (Fig 2.3). These N conditions allowed significant growth of *E. coli* YMC10 and a slightly faster growth was observed under N-rich conditions.

2.3.2 Regulation of *C. acetobutylicum glnA* gene expression by N levels in *E. coli* YMC11. The *E. coli* YMC11 strain is a *glnALG* deletion mutant (Chen et al., 1982) where the *glnA* structural gene, the *glnL* (*ntrB*) and the *glnG* (*ntrC*) regulatory genes have been deleted. Therefore, regulation of the *C. acetobutylicum glnA* expression by N levels was studied in *E. coli* YMC11 as an heterologous host to elucidate the regulatory gene(s) involved in the *C. acetobutylicum glnA* region. *E. coli* YMC11 cells were transformed with plasmid constructs containing

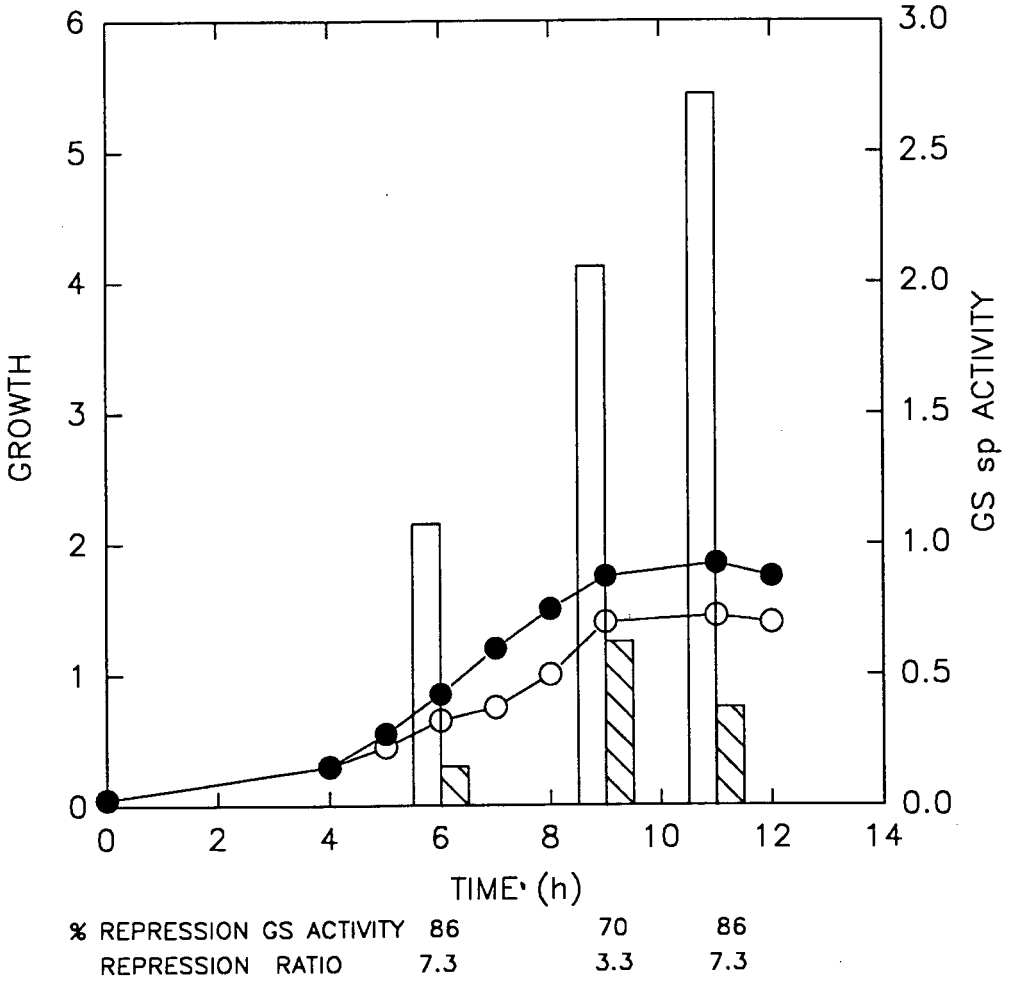


Fig. 2.3. Growth and GS activity of *E. coli* YMC10 cells in N-limiting (M9 medium-15mM gln) (open circles, open bars) and N-rich conditions (M9 medium-15mM gln-0.2% $(NH_4)_2SO_4$) (solid circles, cross hatched bars). GS specific activity was determined at 6, 9, and 11 h and is expressed as micromoles of γ-glutamyl hydroxamate per milligram of protein per min. Each value represents the average of three determinations. Growth is represented as A_{600}. This figure shows the average data of two independent experiments. Percentages of repression of GS activity and repression ratios are shown below the relevant sampling times.

different segments of the C. *acetobutylicum glnA* DNA region (Fig. 2.2) and the expression of GS activity was analyzed under N-rich and N-limiting conditions which supported good cell growth.

Initially, *E. coli* YMC11 cells transformed with pHZ200 (Fig. 2.2), which contained the entire C. *acetobutylicum glnA* region, were grown under the same N-rich and N-limiting conditions used for the growth of the wild type *E. coli* YMC10 strain. GS activity levels were analyzed (Fig. 2.4). Surprisingly, higher levels of GS were produced in the N-rich medium for the first 7 h of growth. After this time interval, a low percentage of repression of GS activity, 12.8% under N-rich compared to N-limiting conditions and a repression ratio of 1.2 were detected when the growth of the cells reached stationary levels. The degree of repression of GS activity in *E. coli* YMC11 (pHZ200) cells after 8 h of growth was minimal in comparison with the repression of GS activity obtained for the wild type *E. coli* YMC10 strain.

2.3.3 Effect of glutamate in the regulation of *C. acetobutylicum glnA* expression. Previous studies on the regulation of C. *acetobutylicum* GS expression in *E. coli* included glutamate (glu) in the growth media (Usdin et al. 1988). In this experiment, glu combined with the N conditions previously described for the growth of *E. coli* YMC10 cells, was used to study the regulation of GS expression in *E. coli* YMC11 cells containing plasmid pHZ200 (Fig. 2.5). The growth of *E. coli* YMC11 cells carrying pHZ200 was determined in all the media and similar growth rates were obtained. When 15mM glu was included in the N-rich and N-limiting growth media (Fig. 2.5) slightly higher percentages of repression of GS activity were observed at 4 and 6 h of growth, compared to the values obtained with cells grown under the same N conditions without glu. However, GS activity repression ratios were low and no marked differences were observed whether

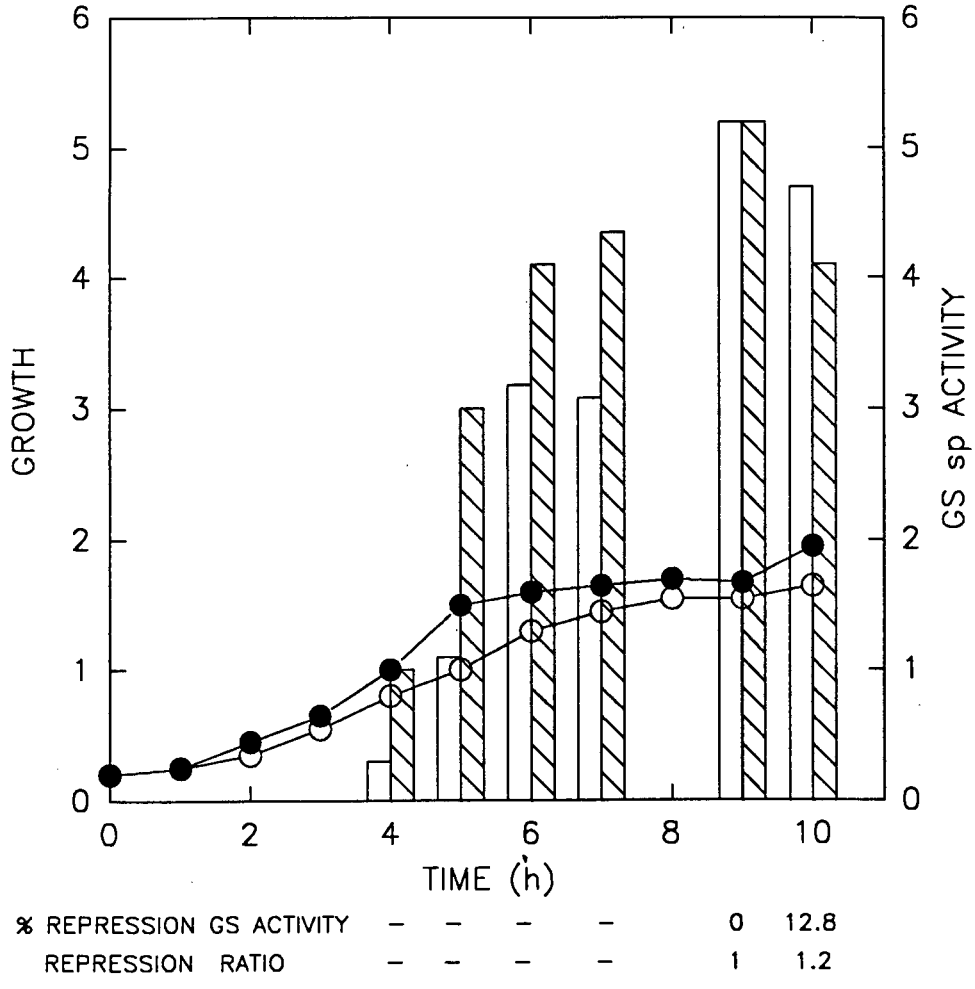
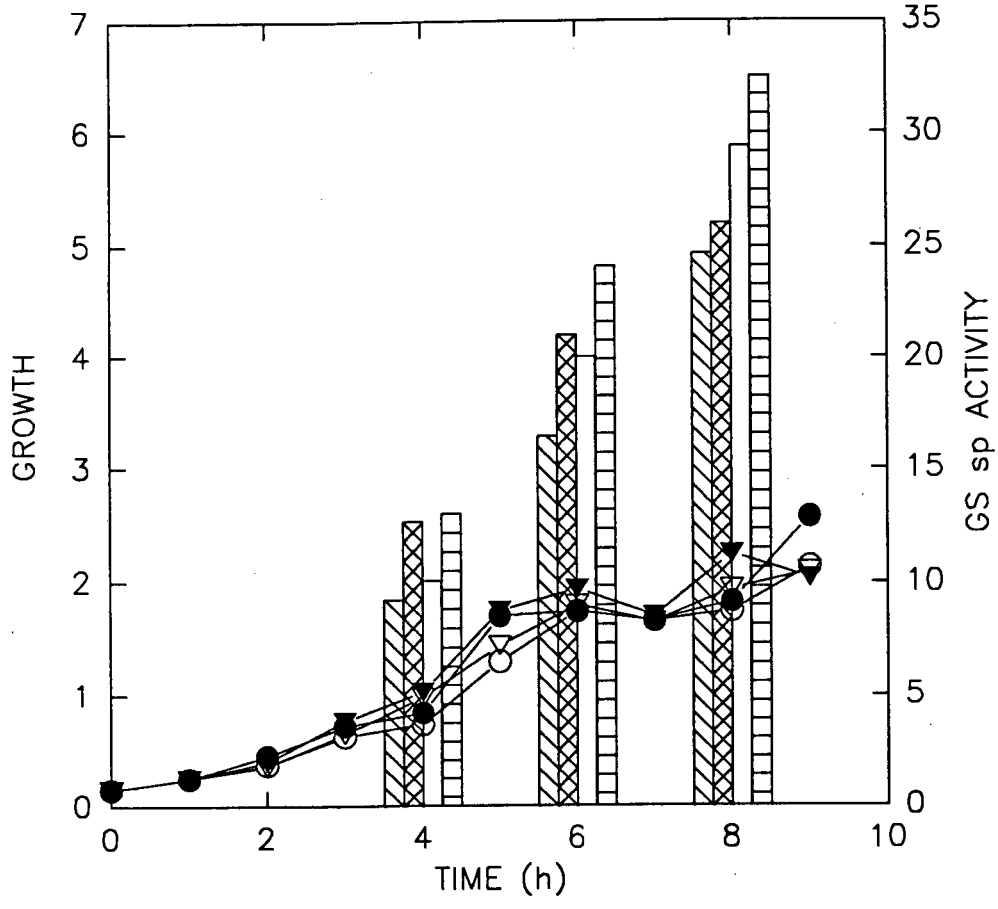


Fig. 2.4. Growth and GS activity of *E. coli* YMC11 (pHZ200) cells in N-limiting (M9 medium-15mM gln) (open circles, open bars) and N-rich conditions (M9 medium-15mM gln-0.2% $(NH_4)_2SO_4$) (solid circles, cross hatched bars). GS specific activity was determined at 4, 5, 6, 7, 9, and 10 h and is expressed as γ-glutamyl hydroxamate per milligram of protein per min. Each value represents the average of three determinations. Growth is represented as A_{600}. This figure shows the average data of two independent experiments. Percentages of repression of GS activity and repression ratios are shown below the relevant sampling times.



	(+)(−)	(+)(−)	(+)(−)
% REPRESSION GS ACTIVITY	8.6 2.7	18 17	16.2 20
REPRESSION RATIO	1.1 1.03	1.2 1.2	1.2 1.3

Fig. 2.5. Growth and GS activity levels of *E. coli* YMC11 (pHZ200) cells in N-limiting and N-rich media with and without glu. The N-limiting media were M9 medium-15mM gln-15mM glu (open triangles, ▽) and M9 medium-15mM gln (open circles, ○). The N-rich medium were M9 medium-15mM gln-15mM glu-0.2% $(NH_4)_2SO_4$ (solid triangles, ▼) and M9 medium-15mM gln-0.2% $(NH_4)_2SO_4$ (solid circles, ●). GS specific activity was determined at 4, 6, and 8 h and is expressed as micromoles of γ-glutamyl hydroxamate per milligram of protein per min. Each value represents the average of three determinations. Growth is represented as A_{600}. This figure shows the average data of two independent experiments. Percentages of repression of GS activity and repression ratios are shown below the relevant sampling times. The (+) and (-) signs indicated the presence or the absence of glu in the media respectively.

glu was present or not in the respective media. At 8 h of growth, both the GS activity repression ratio and the percentage of repression under N-rich compared to N-limiting conditions were slightly lower when glu was present. The presence of glu in the growth media did not have a marked effect on the repression of the GS activity levels.

2.3.4 Involvement of the *C. acetobutylicum glnA* downstream DNA region in the regulation of *glnA* gene expression by N levels. Initially, two plasmids pHZ200 and PHZ202 were analyzed in *E. coli* YMC11. Plasmid pHZ200 contained the whole *C. acetobutylicum glnA* region, and pHZ202 included the entire *glnA* region with an approximately 2.5kb deletion of the DNA sequence downstream of the *glnA* (Fig. 2.2). This plasmid contained 1.3kb of DNA upstream of the *glnA* gene, and included the *glnA* downstream DNA region involving the p_3 promoter, the AS RNA coding sequence and the palindromic DNA (Fig. 2.2). *E. coli* YMC11 transformed with either plasmid pHZ200 (Fig. 2.6.1) or pHZ202 (Fig. 2.6.2) were grown under growth conditions that were more limited for N than the previously described experiments. N-rich conditions involved M9 minimal medium supplemented with 4mM gln and 0.2% $(NH_4)_2SO_4$ and N-limiting conditions contained M9 minimal medium supplemented with 4mM gln. The growth of each strain was monitored under these N conditions and higher growth rates were noticed in N-rich medium. As was observed in the previous experiment, higher GS activity levels were produced under N-rich than under N-limiting conditions at least for the first 6 h of growth. This was detected with either *E. coli* YMC11 (pHZ200) or *E. coli* YMC11 (pHZ202) (Fig. 2.6.2). In these *E. coli* strains, both the repression ratios

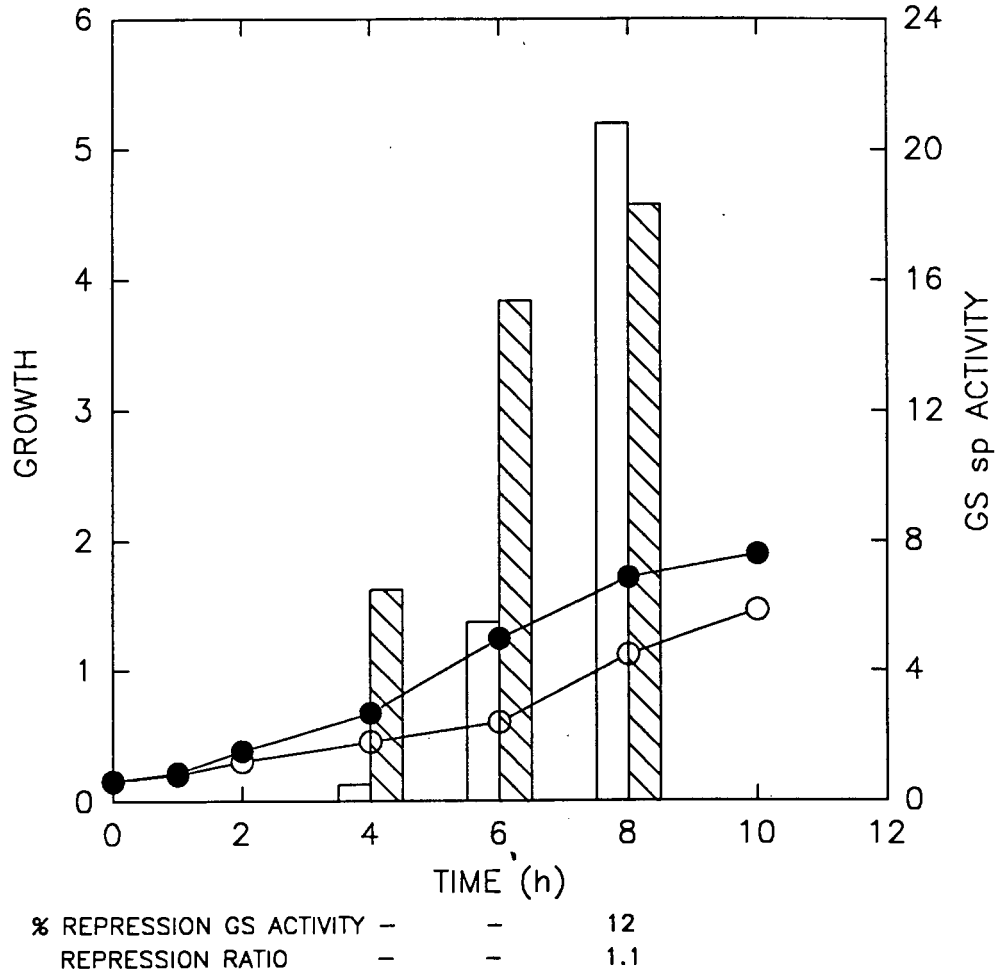


Fig. 2.6.1 Growth and GS activity of *E. coli* YMC11 (pHZ200) cells in N-limiting (M9 medium-4mM gln) (open circles, open bars) and N-rich conditions (M9 medium-4mM gln-0.1% $(NH_4)_2SO_4$) (solid circles, cross hatched bars). GS specific activity was determined at 4, 6, and 8 h and is expressed as micromoles of γ-glutamyl hydroxamate per milligram of protein per min. Each value represents the average of three determinations. Growth is represented as A_{600}. This figure shows the average data of two independent experiments. Percentages of repression of GS activity and repression ratios are shown below the relevant sampling times.

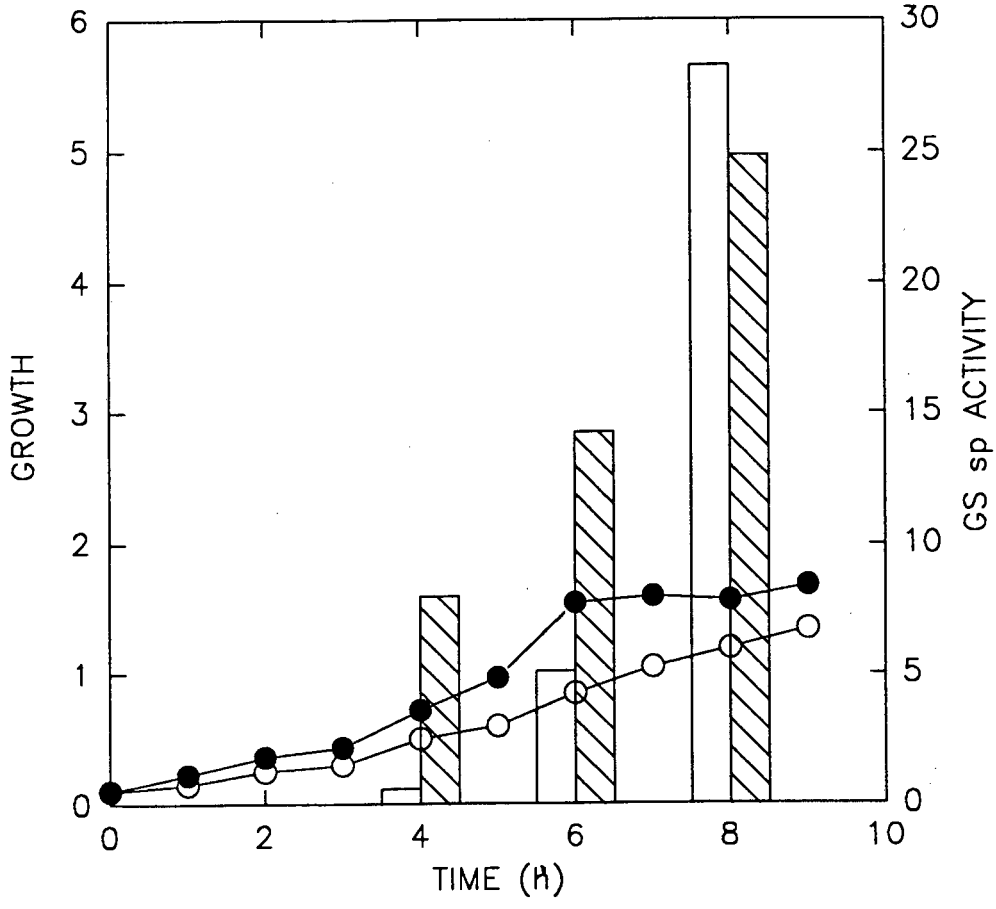


Fig. 2.6.2 Growth and GS activity of *E. coli* YMC11 (pHZ202) cells in N-limiting (M9 medium-4mM gln) (open circles, open bars) and N-rich conditions (M9 medium-4mM gln-0.1% $(NH_4)_2SO_4$) (solid circles, cross hatched bars). GS specific activity was determined at 4, 6, and 8 h and is expressed as micromoles of γ-glutamyl hydroxamate per milligram of protein per min. Each value represents the average of three determinations. Growth is represented as A_{600}. This figure shows the average data of two independent experiments. Percentages of repression of GS activity and repression ratios are shown below the relevant sampling times.

(1.1 for each strain) and the percentages of repression of GS activity (12% for each strain) under N-rich compared to N-limiting conditions were very similar at 8 h of growth. These GS repression values represent very small variations in the GS activity when the respective cells were grown under different N-conditions. Deletion of the approximately 2.5kb region of DNA downstream of p_3 therefore, did not affect the expression of *C. acetobutylicum glnA* gene in *E. coli* YMC11 (compare Figs. 2.6.1 and 2.6.2).

2.3.5 Expression of *C. acetobutylicum* antisense RNA in *E. coli*. To analyze the role of the p_3 promoter and the AS RNA coding sequence in the regulation of the *C. acetobutylicum glnA* gene, *E. coli* YMC11 cells containing pGln1300 or pDR7 were grown under N-limiting conditions (M9 minimal medium supplemented with 4mM gln). *E. coli* YMC11 cells transformed with pGln1300, which contained the *glnA* region including promoter p_3 and the AS RNA coding region, produced between 15.5% and 28.4% less GS activity than the same cells containing pDR7 (Fig. 2.7), which has the *glnA* gene but lacks promoter p_3 and the AS RNA coding region (Fig. 2.2).

When the GS activity of *E. coli* YMC11 cells containing pGln1300 or pDR7 was determined in cells grown in N-rich media, the *E. coli* YMC11 cells containing pGln1300 produced approximately two-fold less GS activity in N-rich conditions (LB medium) compared to the same cells transformed with pDR7 (Fig. 2.8). The difference in GS activity in cells containing pGln1300 or pDR7 was much greater than in N- limiting conditions (200% versus 28%). AS RNA was detected by the ribonuclease protection assay in *E. coli* YMC11 cells containing pGln1300 but was not present in the same cell transformed with pDR7 (Fig. 2.8) (Fierro-Monti et

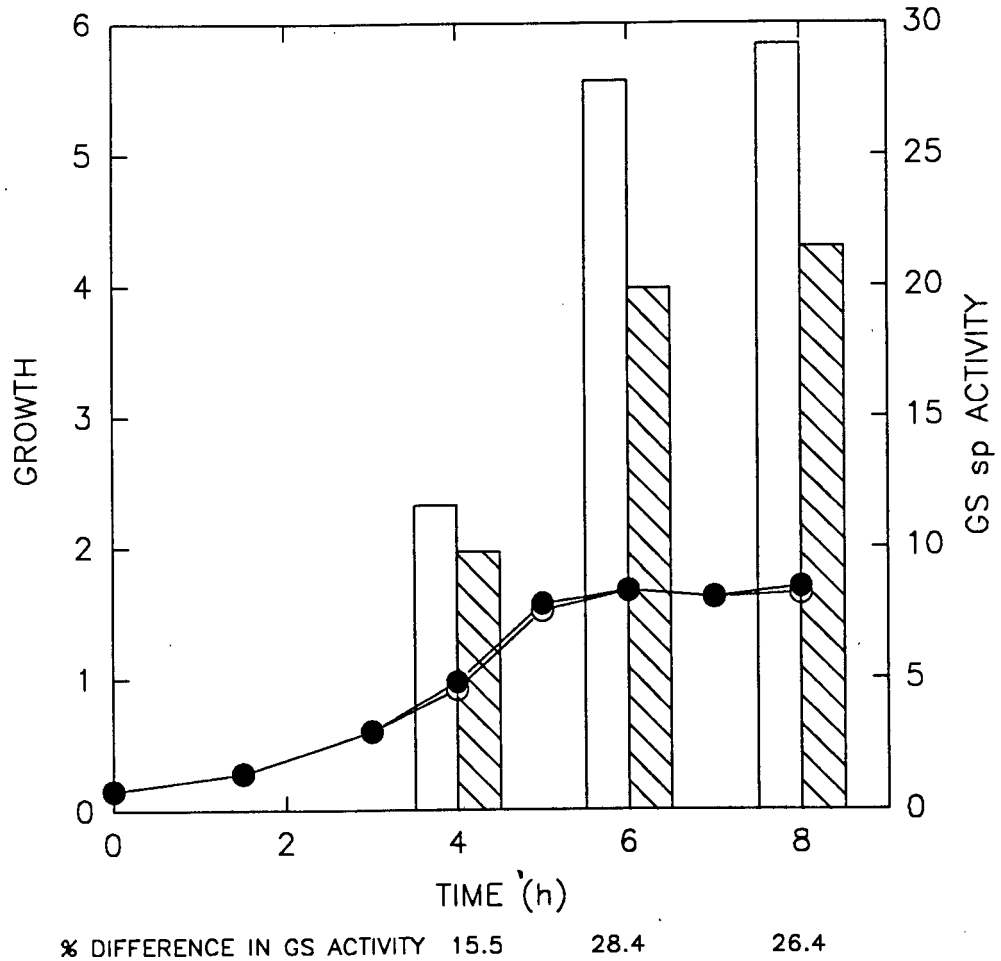


Fig. 2.7. Growth and GS activity of *E. coli* YMC11 (pDR7) (open circles, open bars) or *E. coli* YMC11 (pGln1300) (solid circles, cross hatched bars) in N-limiting conditions (M9 medium-4mM gln). GS specific activity was determined at 4, 6 and 8 h and is expressed as micromoles of γ-glutamyl hydroxamate per milligram of protein per min. Each value represents the average of three determinations. Growth is represented as A_{600}. This figure shows the average data of two independent experiments. Percentages of difference in GS activity between both strains are shown below the relevant sampling times.

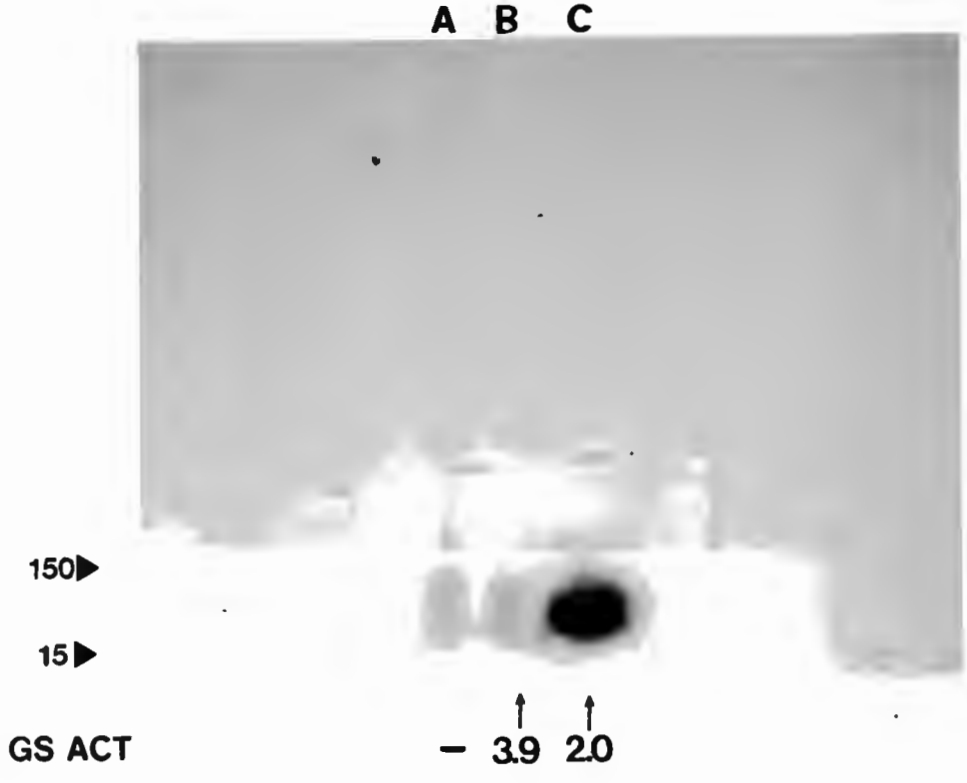


Fig. 2.8. Expression of AS RNA and GS specific activity in exponential phase *E. coli* YMC11 (pGln 1300) and *E. coli* YMC11 (pDR7) cells grown in LB medium. Cultures were harvested at an optical density at 660 nm of 1.0. Total RNA was prepared from *E. coli* YMC11 (lane A), *E. coli* YMC11 (pDR7) (lane B), and *E. coli* YMC11 (pGln 1300) (lane C). The probe used was generated from pSPAS, hybridized with total RNA, and digested with RNases A and T. The protected fragments were separated by 8M urea-5% polyacrylamide gel electrophoresis at 250V. Sizes (bases) of the standard DNA fragments are indicated on the left. GS specific activity is expressed as micromoles of γ-glutamyl hydroxamate per milligram of protein per min; each value represents the average of three determinations.

al., 1992). The presence of the AS RNA in *E. coli* (pGln1300) cells but not in *E. coli* (pDR7) and the lower levels of GS activity in *E. coli* (pGln1300) cells are in agreement with the hypothesis that the AS RNA is interacting with the *glnA* mRNA and inhibiting translation of the *glnA* gene.

The putative involvement of the AS RNA in the regulation of the expression of the *C. acetobutylicum glnA* gene was determined in another experiment in which additional copies of an AS RNA were produced *in trans* by subcloning p_3 and the AS region onto a compatible high copy number plasmid.

coli YMC11 cells containing the entire *glnA* region, including promoter p_3 and the AS RNA coding region on a low copy number plasmid (pTS-1) (Fig. 2.1) produced between 30.4% and 40.4% less GS activity when additional copies of promoter p_3 and the AS RNA coding region were introduced on a high copy number plasmid pJP4 (Fig. 2.9). In this experiment *E. coli* YMC11 cells containing pTS-1 (entire *glnA*), and pJP4 (p_3, AS) or pUC13 (control) were grown in N-limiting conditions (M9 minimal medium supplemented with 4mM gln). These results support the involvement of the AS RNA in the inhibition of GS expression in *E. coli* cells.

2.3.6 Role of Ribonuclease III in the inhibition of *glnA* gene expression by antisense RNA. To determine whether RNaseIII was involved in the repression of *glnA* activity in *E. coli*, pGln1300 and pDR7 were separately transformed into *E.coli* AB301-105, a strain carrying the *rnc-105* mutation and therefore lacking RNaseIII, and *E. coli* YMC11. No difference was observed in GS activity in the RNaseIII-defective strain carrying either plasmid pGln1300 or pDR7 (Table 2.1). An approximately two-fold difference in GS activity was

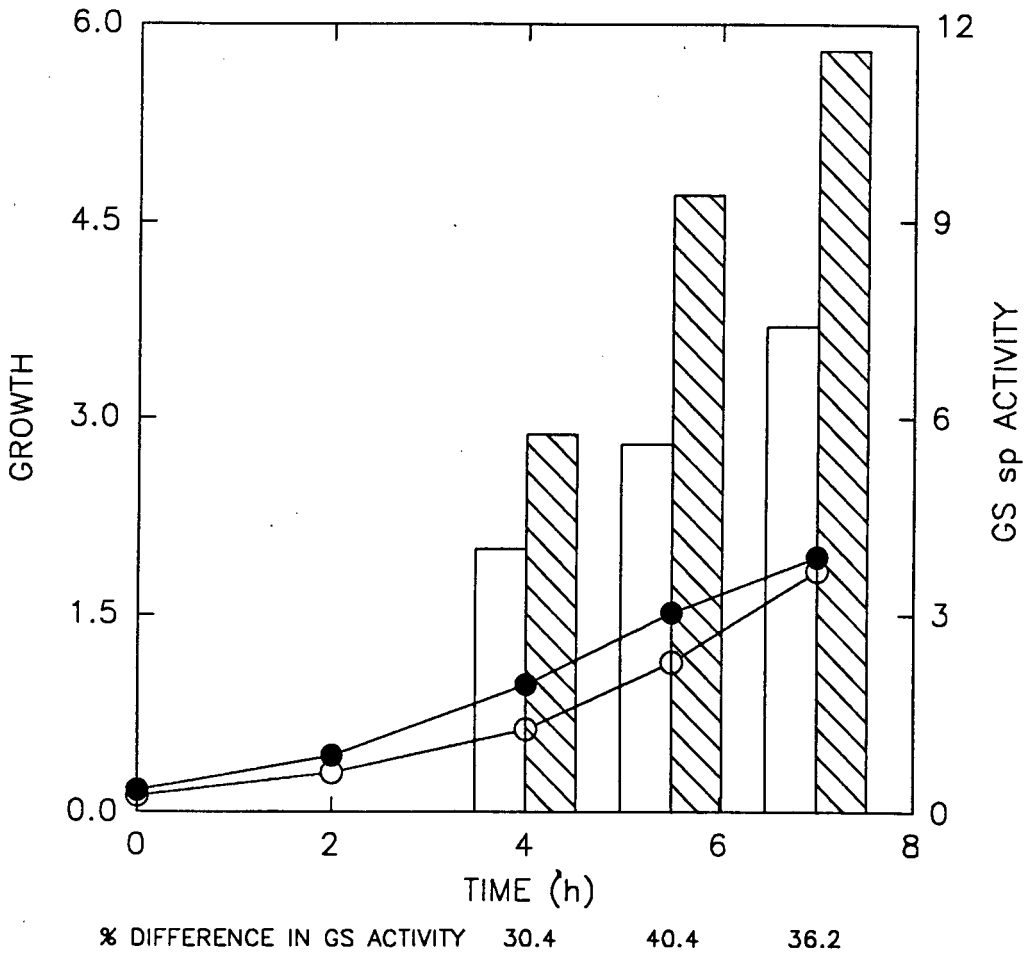


Fig. 2.9. Growth and GS activity of *E. coli* YMC11 containing plasmids pTS-1 and pJP4 (open circles, open bars) or plasmids pTS-1 and pUC13 (solid circles, cross hatched bars) in N-limiting conditions (M9 medium-4mM gln). GS specific activity was determined at 4, 5.5, and 7 h and is expressed as micromoles of γ-glutamyl hydroxamate per milligram of protein per min. Each value represents the average of three determinations. Growth is represented as A_{600}. This figure shows the average data of two independent experiments. Percentages of difference in GS activity between both strains are shown below the relevant sampling times.

detected between *E. coli* YMC11 (pGln1300) and *E. coli* YMC11 (pDR7), when RNaseIII was present in these strains (Table 2.1). This suggests that in *E. coli*, RNaseIII plays an essential role in the repression of *C. acetobutylicum glnA* expression presumably by digestion of the AS RNA-*glnA* hybrid. Further *in vitro* experiments are required to demonstrate specific degradation of the RNA hybrid by RNase III. The participation of RNaseIII activity in the cleavage of the AS RNA-target mRNA hybrid to produce an irreversible inhibition of the mRNA expression has been demonstrated in several AS control systems (Krinke and Wulff, 1987).

Table 2.1. Expression of GS activity of the *C. acetobutylicum glnA* gene in *E.coli* AB301 -105 and in *E.coli* YMC11.

Strain	GS Activity [a]
E. coli AB301-105 (pGln1300)	1.9
E. coli AB301-105 (pDR7)	2.0
E. coli AB301-105	0.03
E. coli YMC11 (pGln1300)	2.0
E. coli YMC11 (pDR7)	3.9
E. coli YMC11	N.D. [b]

[a] GS activity was measured at two times interval during exponential growth in LB medium. Activity is expressed as micromol of γ-glutamyl hydroxamate/mg of protein per min. Each value represents the average of three determinations.

[b] N.D.- not detectable.

2.3.7 CONCLUSION

Although previous results (Usdin et al., 1988; Janssen et al., 1988; 1990) indicated that GS expression in this host was regulated by N levels under specific N conditions tested, no significant differences in GS activity levels were obtained in these studies under N conditions which allowed significant growth of the respective cells. Different plasmid constructs carrying deletions of the *C. acetobutylicum glnA* region were tested for GS regulation under similar N conditions, however no significant GS regulation by N levels was observed when these constructs were present in the heterologous *E. coli* YMC11 host.

GS activity levels in *E. coli* YMC11 transformed by a plasmid construct carrying the entire *glnA* region compared to the same cells carrying a construct in which this AS RNA coding sequence was deleted resulted in 33% or two-fold less GS activity in N-limiting or N-rich conditions, respectively. The presence of the AS RNA transcript was demonstrated by RPA analysis in *E. coli* YMC11 cells containing a construct with the AS RNA coding sequence (Fierro-Monti et al., 1992). The inhibitory *in trans* effect of the AS RNA on the GS activity was demonstrated when *E. coli* YMC11 cells carrying the *glnA* region (present in a low copy number vector) and additional copies of the AS RNA coding sequence (in a high copy number vector) produced 25% less GS activity when these cells where grown in N-limiting conditions.

CHAPTER THREE

REGULATION OF THE *glnA* GENE AND ANTISENSE RNA EXPRESSION IN *C. ACETOBUTYLICUM.*

SUMMARY: The development of suitable media for the growth of *C. acetobutylicum* cells allowed the analysis of the regulation of the *glnA* gene expression in the homologous host. Expression of the 43-base *glnA* AS RNA was demonstrated in *C. acetobutylicum.* In these cells, GS activity, the transcription of *glnA* mRNA and the *glnA* AS RNA were regulated by N. GS activity and *glnA* mRNA were repressed in cells grown in N-rich medium. Repression ratios for GS activity varied from 1.6 to 9.0, depending on the sampling time. The relative number of *glnA* transcripts was approximately 25%-28% lower in cells grown for 72 h in N-rich medium than in cells grown in N-limiting medium. This finding contrasted with the expression of the *glnA* AS RNA, which was repressed in N-limiting medium but induced in N-rich medium. The relative number of AS RNA transcripts was increased approximately 1.35 to 6-fold in cells grown in N-rich medium. There was 1.48 to 1.6-fold excess of AS RNA over *glnA* mRNA under conditions that repressed GS activity. Under conditions that induced GS activity, *glnA* mRNA transcripts exceeded AS RNA transcripts by 1.3 to 5-fold.

3.1. INTRODUCTION

Mechanisms of the control of gene expression have most often involved regulatory proteins that either activate or repress the expression of specific gene products. The role of AS RNA in the control of gene expression is relatively recent. An increasing number of biological systems have been found to be affected by this control mechanism. Some of these systems which occur in bacteria have been described previously (Chapter 1) and only a few bacterial chromosomal genes have been demonstrated to be controlled by AS RNA. Among these genes are *ompF, crp, sulA, ftsZ,* and *gnd* (Mizuno et al., 1984; Okamoto and Freundlich, 1986; Cole and Honore, 1989; Faubladier et al., 1990; Carter-Muenchau and Wolf, 1989). Most of these genes are characterized by being regulated in response to environmental or to intracellular signals, including changes in osmolarity, temperature, metabolic state, and cell division.

N-limitation is an important external signal sensed by the cell. This signal is communicated through transducing pathways that can switch on the expression of regulatory genes or sequences which control different N metabolism related genes. An increase in the expression of GS levels as a result of N limitation is observed in a number of prokaryote microorganisms. This increase is normally accompanied by an enhancement in the expression of genes participating either in the assimilation or the catabolism of N compounds.

Two major pathways are involved in ammonia assimilation; the GDH pathway and the GS/GOGAT pathway. Studies have been focused on the regulation of *C. acetobutylicum glnA* gene expression and a recent analysis has implicated a distinctive control system (Fierro-Monti et al., 1992). Studies on the structure of the *C. acetobutylicum glnA* region in *E. coli* YMC11 (a *glnALG* deletion mutant) revealed the presence of a putative AS RNA transcript complementary to the

5'start and ribosome binding site of the *glnA* gene. Further studies on the expression of the C. *acetobutylicum glnA* AS RNA in *E. coli* linked the presence of AS RNA molecules with decreased levels of GS activity (Chapter 2). Since *E. coli* was a heterologous host is important that the expression of the *glnA* gene and the *glnA* AS RNA is investigated in C. *acetobutylicum.*

Since a colleague in our laboratory had cloned a putative *gltS* gene coding for the small subunit of GOGAT in the C. *acetobutylicum* host (H. Stutz, PhD thesis) it was also important to investigate the expression of this gene in relation to the *glnA* gene. In prokaryotes, the GOGAT enzyme occurs as a multimer of heterodimer complexes. The *E. coli* GOGAT enzyme is formed by a large and a small subunit encoded by *gltB* and *gltD* genes, respectively. DNA sequencing of a plasmid containing a 1.63kb DNA region of C. *acetobutylicum* has shown the presence of an ORF of 420 aa with homology (56% similarity) to the *E. coli* small subunit of GOGAT (Helen Stutz, PhD thesis).

3.2 MATERIALS AND METHODS

3.2.1. Bacterial strains and growth conditions. *C. acetobutylicum* strain P262 (Jones et al., 1982) (Apendix B.3) was grown under anaerobic conditions in *Clostridium* basal medium (O'Brien et al., 1971) or glucose-mineral salts-biotin medium (GSMM) (Holdeman et al., 1977) modified as follows: the published N source was omitted, and the N-free medium was supplemented with 0.1% gln (N-limiting medium) or with 0.1% gln plus 0.1% $(NH_4)_2SO_4$ (N-rich medium).

3.2.2. Preparation of nucleic acid. Plasmid DNA was prepared by the method of Ish-Horowicz and Burke, 1981 (Appendix A.1.1-2). RNA from *C. acetobutylicum* was extracted by the method of Aiba et al., (1981) (Appendix A.1.12), and stored at -70°C in 70% ethanol.

3.2.3. Radiolabelling of DNA probe. Isolated *C. acetobutylicum* DNA fragments were nick translated (Rigby et al., 1977) with [α-^{33}P] dATP or dCTP using the nick translation kit of Amersham International (code N.5500). Contaminating nucleotides were removed from the radioactively labeled probe solution by using a Sephadex G50 spin column as described by Maniatis et al., (1984). The radioactive probes were stored in lead containers at -20°C.

3.2.4. DNA hybridization. Standard procedures with minor modifications were followed and are described in Appendix A.1.17.

3.2.5. Plasmid constructions. Plasmid pSPAS and pSPgln700 have been described in Chapter 2. Plasmid pglt78 (Appendix B.2) contained a 490 nucleotide DNA fragment of the putative *C. acetobutylicum gltS* gene encoding the 5'end of the putative small subunit of GOGAT subcloned into pBluescript-SK

(Helen Stutz, PhD thesis). The transcription of this DNA insert was under the control of the T3 promoter sequence, and the T3 polymerase was utilized for the synthesis of the riboprobe by *in vitro* transcription assays (Appendix A.1.13.2). These plasmids were used to generate single-stranded RNA probes for the RNase protection assay. Nucleotide sequences of plasmid constructs were confirmed by sequence analysis as described in Chapter 4.

3.2.6. RNase protection assay. The ribonuclease protection assay was used for the detection and quantitation of RNA species. This method has been described in Chapter 2 and in Appendix 1.14.1-3.

3.2.7. Molecular titration assay. The previously described single stranded RNA probes (Chapter 2 and Appendix A.1.15) were used in tracer-excess titrations to quantify the levels of *glnA* mRNA, AS RNA transcripts and the putative *gltS* mRNA in *C. acetobutylicum* (Lee and Costlow, 1987). Measurements were carried out on total RNAs prepared from *C. acetobutylicum* P262 cells grown for 72 h in N-limiting (0.1% gln) and N-rich [0.1% gln plus 0.1% $(NH_4)_2SO_4$] or [0.1% $(NH_4)_2SO_4$] media. The titration assay consisted of a series of hybridizations between a constant amount of the RNA probe in excess of the target RNA species and increasing amounts of input cellular RNA. Hybridizations and RNase digestions of the protected fragments were performed as described for the RNase protection assay (Appendix 1.14.1-3); these procedures were followed by precipitation in 0.5 M sodium phosphate buffer, pH 7.0. The data were expressed as RNase-resistant counts per min versus total RNA input. The slopes of the lines from the data were used to calculate the relative transcript prevalence of the *glnA* mRNA, *glnA* AS RNA and putative *gltS* mRNA, after correcting for specific activity and the length of the hybridizable portion of each RNA probe (Appendix 1.13.5).

3.2.8. Enzyme assays. GS activity was determined as described in Chapter 2 by the γ-glutamyl transferase assay according to Shapiro and Stadtman, 1968 (Appendix A.1.10). Percentages of repression/difference, and repression ratios of GS activity were calculated as before (Chapter 2).

3.3. RESULTS AND DISCUSSION

3.3.1. Development of a minimal medium for *C. acetobutylicum* cell growth. Analysis of GS expression in *C. acetobutylicum* required the development of a minimal medium containing either limiting or rich N levels. GSMM supplemented with different N sources was utilized to grow *C. acetobutylicum* cells. GS activity levels were determined in the cells grown in the various media (Fig. 3.1).

Growth of the cells in GSMM supplemented with gln was relatively fast during the exponential growth phase and reached the highest yields. In GSMM supplemented with ammonium sulfate, cell growth was slower during the exponential phase than in the previous medium, and lower cell yields were obtained. Growth in GSMM supplemented with glu was a little faster during the exponential phase when compared to the growth in GSMM supplemented with ammonium acetate, although similar low cell yields were obtained in both these media (Fig 3.1).

Levels of GS activity detected in cells grown in media containing glu or ammonia (ammonium sulfate or ammonium acetate) were lower compared to the GS activity levels detected in media containing gln. Therefore, growth of *C. acetobutylicum* cells in GSMM supplemented with gln produced the highest GS activity levels (Fig. 3.1).

3.3.2. Regulation of GS activity in *C. acetobutylicum*. To study the expression of the *C. acetobutylicum glnA* gene, cells were grown in N-limiting (GSMM-0.1% gln) and N-rich (GSMM-0.1%gln-0.1% $(NH_4)_2SO_4$) media. High levels of GS

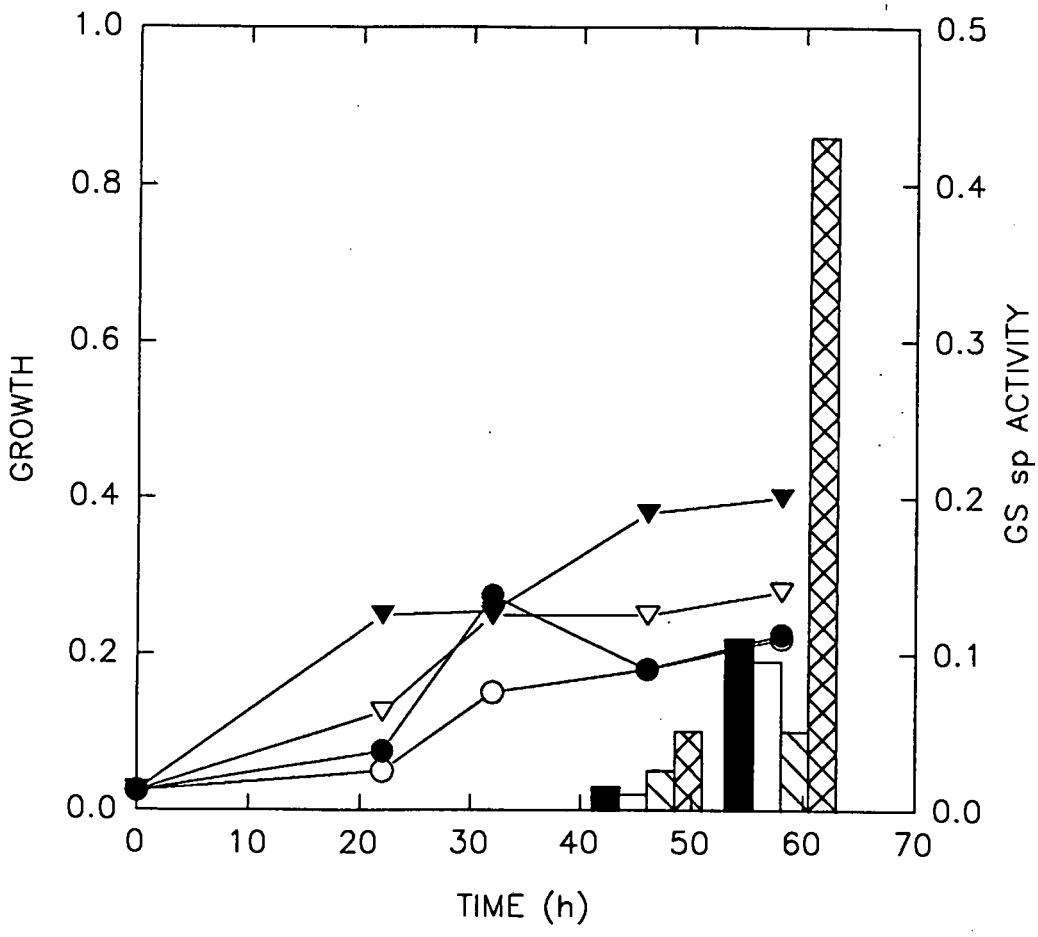


Fig. 3.1. Growth and GS activity of *C. acetobutylicum* cells in GSMM supplemented with different N sources. (solid triangles, ⊠) 0.1% gln, (open triangles, ▨) 0.1% ammonium sulfate, (solid circles, ▩) 0.1% glu, and (open circles,□) 0.1% ammonium acetate. GS specific activity was determined at 46 and 58 h and is expressed as micromoles of γ-glutamyl hydroxymate per milligram of protein per min. Each value represent the average of three determinations. Growth is represented as A_{600}. This figure shows the average data of two independent experiments.

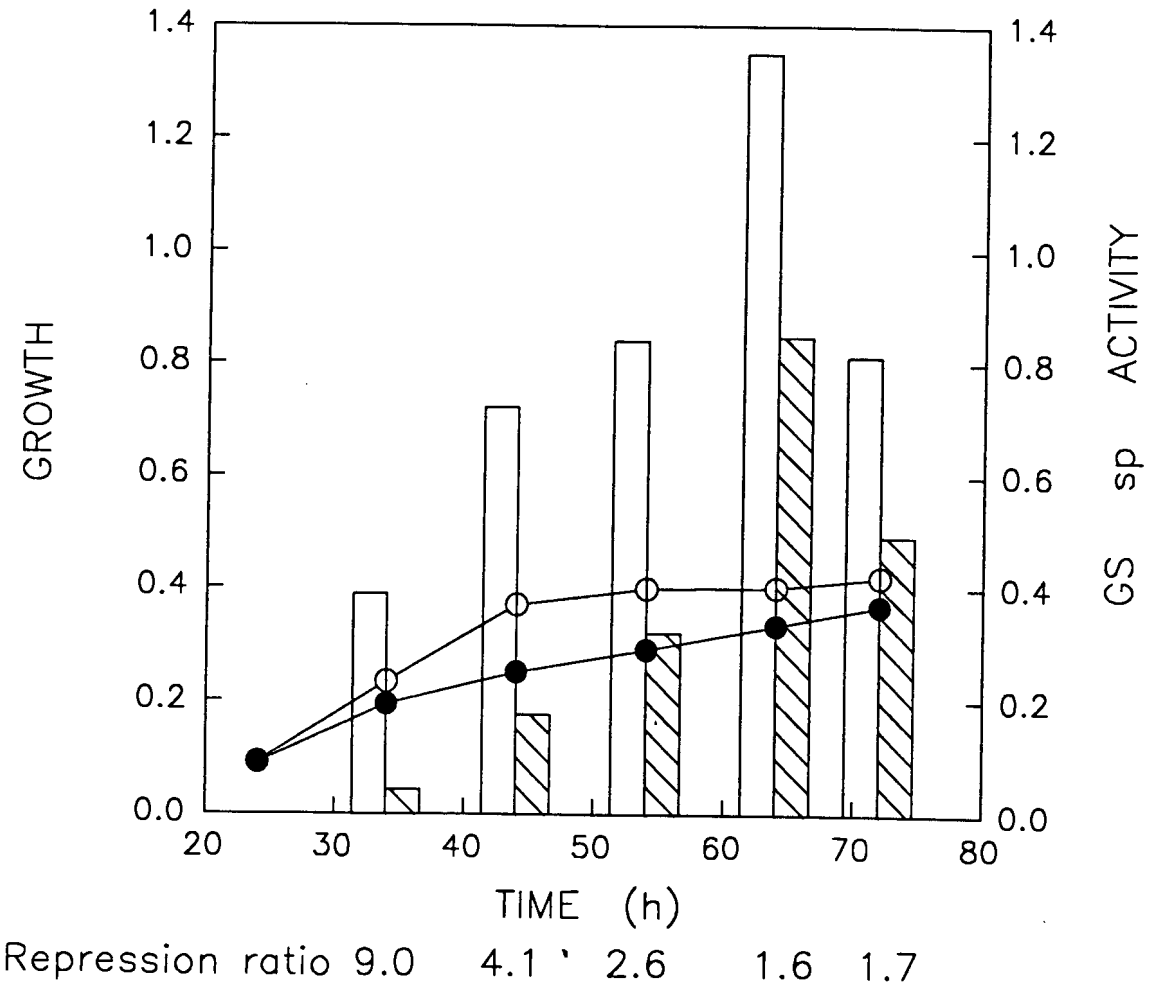


Fig. 3.2. Growth and GS activity of *C. acetobutylicum* cells in GSMM-0.1%gln (open circles, open bars) and GSMM-0.1%gln-0.1%$(NH_4)_2SO_4$ (solid circles, cross-hatched bars) (Fierro-Monti et al., 1992). GS specific activity was determined at 34, 44, 54, 64, and 72h and is expressed as micromoles of γ-glutamyl hydroxymate per milligram of protein per min. Each value represents the average of three determinations. Growth is represented as A_{600}. This figure shows the average data from two independent experiments. Repression ratios are shown below the relevant sampling times.

activity were obtained in *C. acetobutylicum* cells grown under N-limiting conditions (Fig. 3.2). Growth of *C. acetobutylicum* cells in N-rich media resulted in repression ratios which varied from 1.6 to 9.0, depending on the sampling time (Fierro-Monti et al., 1992).

3.3.3. Identification of the *glnA* antisense RNA in *C. acetobutylicum*. Plasmid pSPAS was previously described in Chapter 2 (Fig. 2.2). This plasmid contained *C. acetobutylicum* DNA of approximately 700bp including the AS DNA region subcloned downstream of the SP6 promoter in plasmid pSP64. Plasmid pSPAS was used for the synthesis of a single-stranded RNA probe complementary to the AS RNA. RNase protection assays using this RNA identified a protected RNA fragment in *C. acetobutylicum* cells (Fig. 3.3a) (Fierro-Monti et al., 1992). Molecular size determination of the protected AS RNA transcript, using pBR322 digested with *Msp*I endonuclease as standard size markers, indicated that the protected RNA fragment consisted of 43 bases (Fig. 3.3b). The AS RNA probe was specific for the AS RNA and did not hybridize with any other cellular RNA produced by *C. acetobutylicum*.

3.3.4. Identification of the *glnA* antisense RNA coding region in the *C. acetobutylicum* chromosome. Southern blot hybridization of a DNA probe against *C. acetobutylicum* chromosomal DNA and plasmid pHZ200 as a control, was performed. The probe corresponded to a *C. acetobutylicum* DNA fragment that contained the *glnA* AS RNA coding region. This DNA probe consisted of a *Alu*I and *Eco*RV restriction endonuclease digested DNA fragment (Fig. 4.2). The control plasmid pHZ200 contained the entire *C. acetobutylicum glnA* region. The DNA probe hybridized with a *C. acetobutylicum* chromosomal *Hin*dIII DNA fragment of approximately 0.75kb (Fig. 3.4, lane 2) that corresponded to a DNA

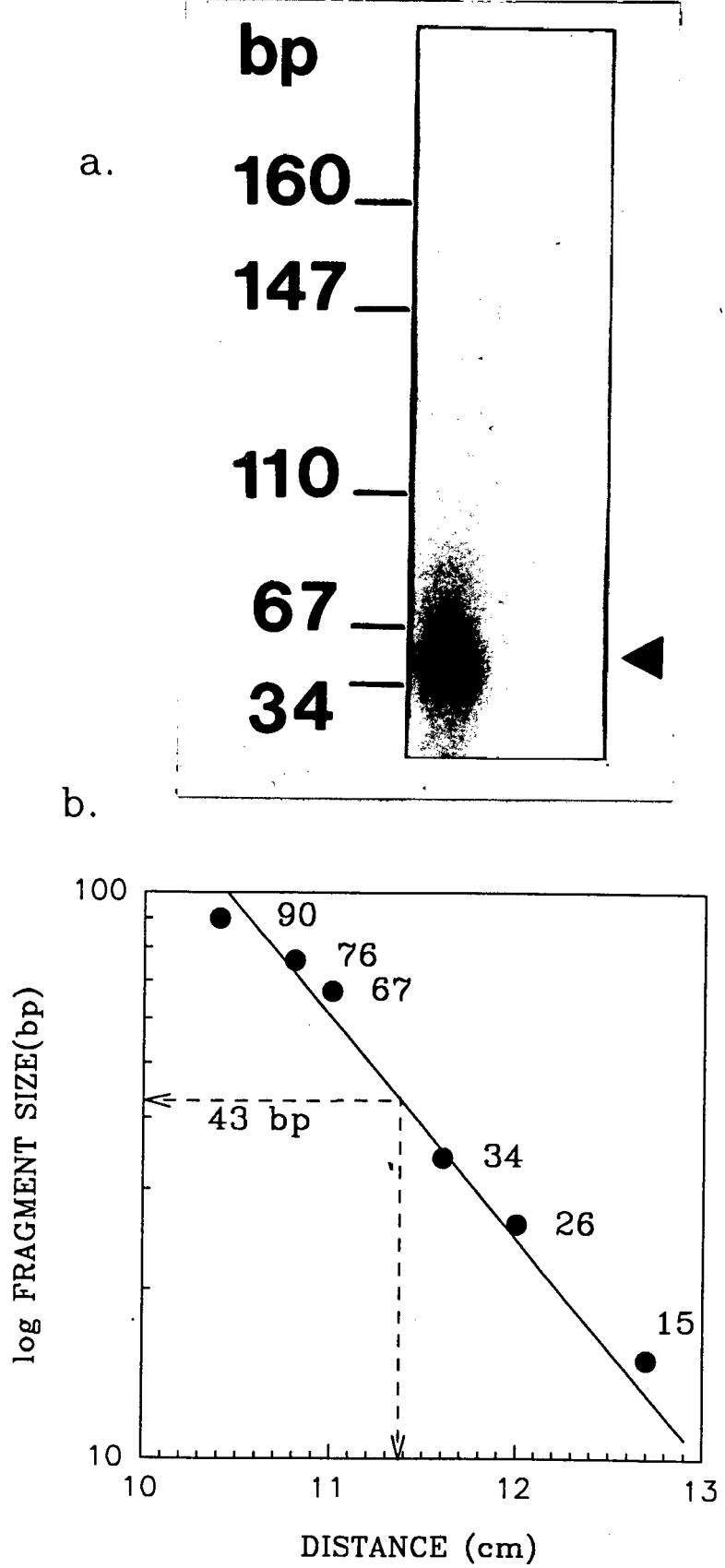


Fig. 3.3. (a) Detection of the *glnA* AS RNA in *C. acetobutylicum* (Fierro-Monti et al., 1992). Total RNA was prepared from cells grown in *Clostridium* basal medium in exponential phase and hybridized with the single stranded RNA probe generated from pASPAS plasmid digested with RNases A and T and separated by an 8 M urea-14% polyacrylamide gel electrophoresis at 250V. pBR322 plasmid DNA digested with *Msp*I was used as the standard DNA size marker. Sizes of the DNA fragments are indicated on the left. The arrowhead shows the protected AS RNA fragment. **(b)** Size determination of the *glnA* AS RNA.

fragment from the control plasmid pHZ200 digested with *Hin*dIII (Fig. 3.4, lane 1), which contained the *glnA* AS RNA coding region. The DNA probe also hybridized with *C. acetobutylicum* chromosomal DNA digested with the endonuclease *Eco*RI and two DNA fragments of approximately 2.7kb (stronger signal) and 2.4kb (weaker signal) (Fig. 3.4, lane 4) were detected. The 2.7kb *Eco*RI DNA fragment corresponded to the 2.7kb *Eco*RI DNA fragment from pHZ200 containing the *glnA* AS RNA coding sequence (Fig. 3.4, lane 3). The signal produced by the 2.4kb fragment (Fig. 3.4, lane 4) may correspond to the DNA region situated upstream of the *Eco*RI restriction site located within the *glnA* gene in the *C. acetobutylicum* chromosome (Fig. 4.2). In the *C. acetobutylicum* chromosome this region is flanked by an internal *Eco*RI restriction site within *glnA* and a *Eco*RI restriction site situated in the region upstream of the *glnA* gene. This region contains the upstream 1.3kb DNA sequence and the 5' start of the *glnA* gene, which is homologous to the AS RNA coding region.

In addition, the DNA probe hybridized with the control plasmid pHZ200 digested with restriction endonucleases *Hin*dIII or *Eco*RI, and DNA fragments of 6.35 or 5.1kb were detected (Fig. 4.2, lanes 1 and 3). Either of these two fragments contained the 5'start region of the *glnA* gene and the rest of the pHZ200 control plasmid.

Therefore, it is concluded that the *glnA* AS RNA coding region is only present in the DNA region downstream of the *C. acetobutylicum glnA* gene within the *C. acetobutylicum* chromosome, and there is high homology between this region and the DNA region situated at the 5'start of the *glnA* gene.

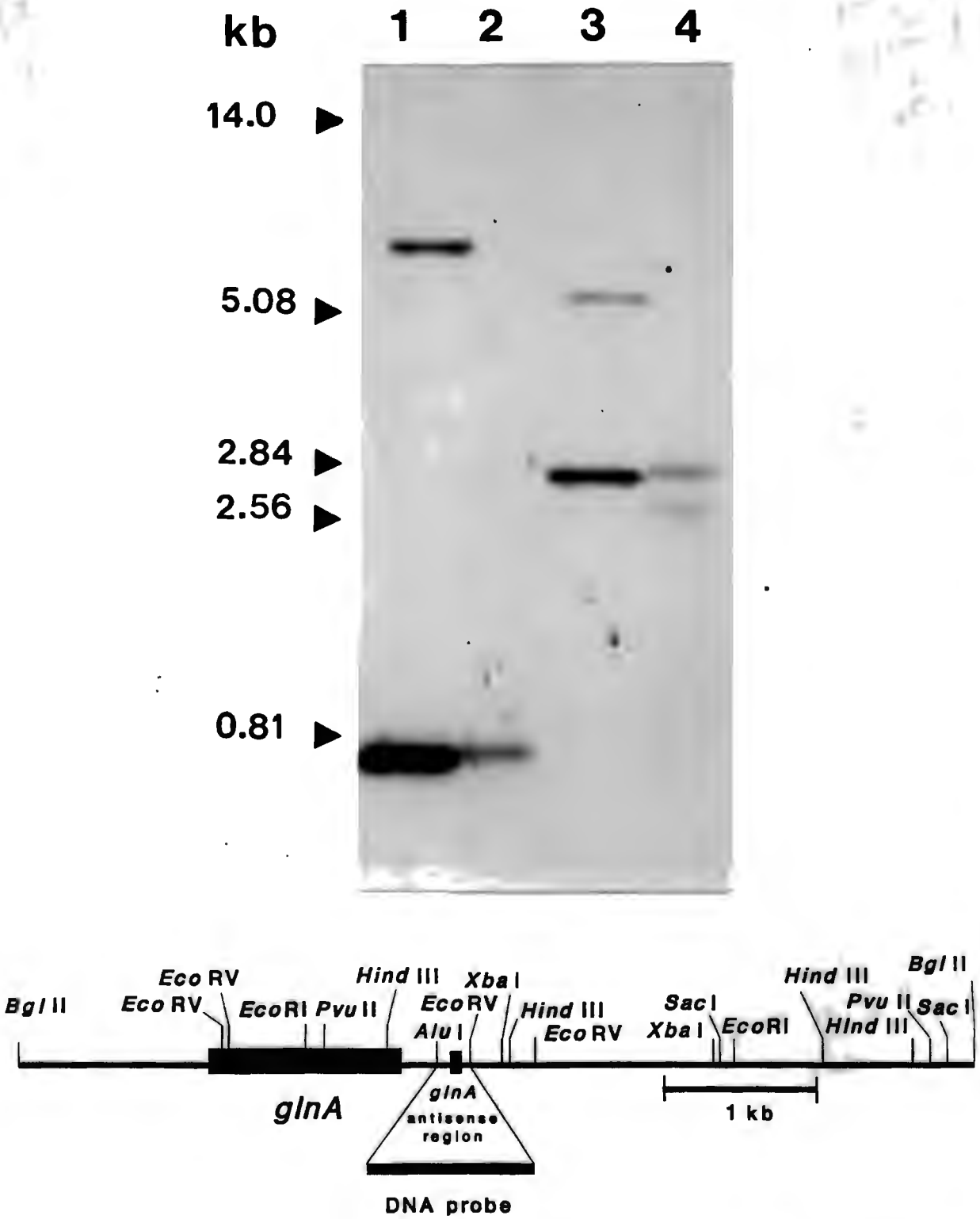


Fig. 3.4. Hybridization of radioactively labelled *Alu*I and *Eco*RV restriction endonuclease digested *C. acetobutylicum* DNA fragment with membrane immobilized *C. acetobutylicum* chromosomal DNA and plasmid pHZ200 (Fig.2.2) as a control. The DNA fragment utilized as a probe and the whole *C. acetobutylicum glnA* region present in plasmid pHZ200 are shown. Lanes 1 and 3, *Hin*dIII and *Eco*RI restriction endonuclease digested plasmid pHZ200, respectively. Lanes 2 and 4, *Hin*dIII and *Eco*RI restriction endonuclease digested *C. acetobutylicum* chromosomal DNA, respectively. Lambda DNA digested with *Pst*I restriction endonuclease was used as standard DNA for size determinations.

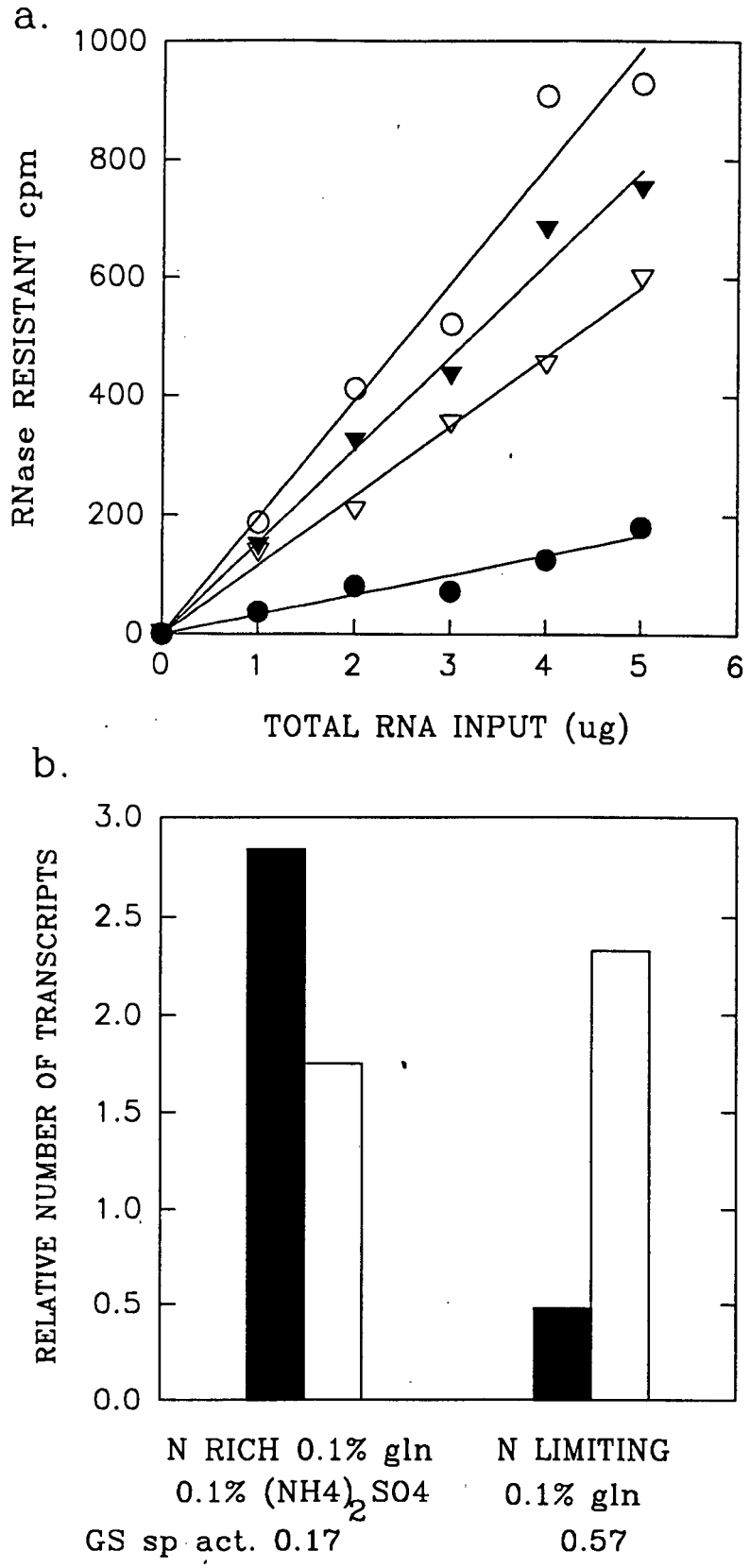


Fig. 3.5. Levels of *glnA* AS RNA, *glnA* mRNA, and GS activity in *C. acetobutylicum* cells grown under N-limiting (GSMM-0.1%gln) or N-rich (GSMM-0.1%$(NH_4)_2SO_4$-0.1%gln) conditions (Fierro-Monti et al., 1992). **(a)** Levels of

AS RNA and mRNA. Total RNA was prepared from cells grown in two independent cultures for 72h under N-limiting (solid triangles, solid circles) or N-rich (open triangles, open circles) conditions and hybridized to a single stranded RNA probe generated from pSPAS or pSPgln700. The quantity of RNA-RNA hybrid for the AS RNA (open circles, solid circles) or *glnA* mRNA (open triangles, solid triangles) was assayed in each sample after digestion with RNaseA and RNaseT. Data are presented as RNase-resistance counts per min versus total RNA input. Each value represents the average of two determinations. **(b)** Relative number of AS RNA and mRNA transcripts and GS specific activity in cells harvested at 72h from N-limiting and N-rich media. The slopes of the lines (shown in panel a) were used to calculate the relative number of AS RNA (solid bars) or *glnA* mRNA transcripts (open bars) under N-limiting or N-rich conditions. GS specific activity was determined and is expressed as micromoles of γ-glutamyl hydroxymate per milligram of protein per min; each value represents the average of three determinations.

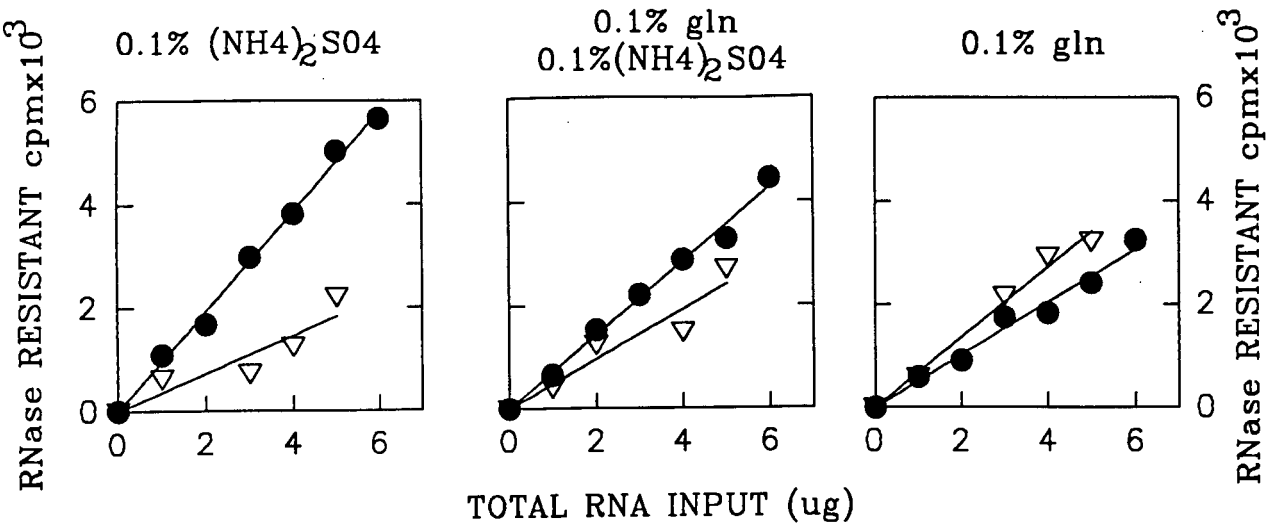
3.3.5. Regulation of GS activity, *glnA* mRNA, and *glnA* antisense RNA transcription in *C. acetobutylicum*. A molecular titration assay based on the RNase protection assay (Lee and Costlow, 1987) was used to determine the transcript prevalence levels of *glnA* mRNA and *glnA* AS RNA in *C. acetobutylicum* cells grown for 72 h in N-limiting (GSMM-0.1% gln) and N-rich (GSMM-0.1%gln-0.1% $(NH_4)_2SO_4$) media (Fig. 3.5). Higher levels of *glnA* mRNA were produced under N-limiting than in N-rich conditions. The relative levels of *glnA* mRNA were reduced by approximately 25% when the cells were grown in N-rich medium (Fig. 3.5).

The transcription of *C. acetobutylicum glnA* AS RNA was differentially regulated by N in comparison with the transcription of *glnA* mRNA (Fig. 3.5) (Fierro-Monti et al., 1992). Six-fold higher levels of *glnA* AS RNA were detected in cells grown in N-rich medium than in cells grown in N-limiting medium. The levels of *glnA* AS RNA relative to the *glnA* mRNA levels was found to be 1.63 in N-rich medium and 0.21 in N-limiting medium (Fig. 3.5b). GS activity was determined in the *C. acetobutylicum* cells from which the mRNA and AS RNA were isolated.

These cells were grown (for 72 h) in N-limiting and N-rich media and the GS specific activity under these conditions was 0.57 and 0.17 micromoles per milligram of protein per min, respectively.

In a similar experiment *C. acetobutylicum* cells were grown in the previous N-limiting (GSMM-0.1% gln) and N-rich (GSMM-0.1% gln-0.1% $(NH_4)_2SO_4$) conditions, and in a third N-rich (GSMM-0.1% $(NH_4)_2SO_4$) medium. A molecular titration assay based on the RNase protection assay was used to determine the transcript prevalence levels of *glnA* mRNA and *glnA* AS RNA in the *C. acetobutylicum* cells grown for 72 h in the three respective media. Higher levels of *glnA* mRNA were produced when the cells were grown under N-limiting compared to the two N-rich conditions (Fig. 3.6). The relative levels of *glnA* mRNA were reduced by approximately 28% or 47% when the cells were grown in N-rich (GSMM-0.1%gln-0.1%$(NH_4)_2SO_4$) or N-rich (GSMM-0.1%$(NH_4)_2SO_4$) media respectively. In this experiment the transcription of *C. acetobutylicum glnA* AS RNA was differentially regulated by N in comparison with the regulation of *glnA* mRNA. An increase of 1.35-fold or 1.8-fold of *glnA* AS RNA was detected in cells grown in N-rich (GSMM-0.1%gln-0.1%$(NH_4)_2SO_4$) or N-rich (GSMM-0.1%$(NH_4)_2SO_4$) media respectively, than in cells grown in N-limiting (GSMM-0.1%gln) medium. The levels of *glnA* AS RNA relative to the levels of *glnA* mRNA were found to be 1.48-fold or 2.69-fold higher in N-rich (GSMM-0.1%gln-0.1% $(NH_4)_2SO_4$) or N-rich (GSMM-0.1% $(NH_4)_2SO_4$) media respectively, and 0.78 fold in N-limiting (GSMM-0.1%gln) medium (Fig. 3.6). GS activity levels were determined in the *C. acetobutylicum* cells grown in these three different media, and the GS specific activity of the cells grown under these three conditions was 0.812, 0.404 and 0.492 micromoles per milligram of protein per min, respectively.

a.



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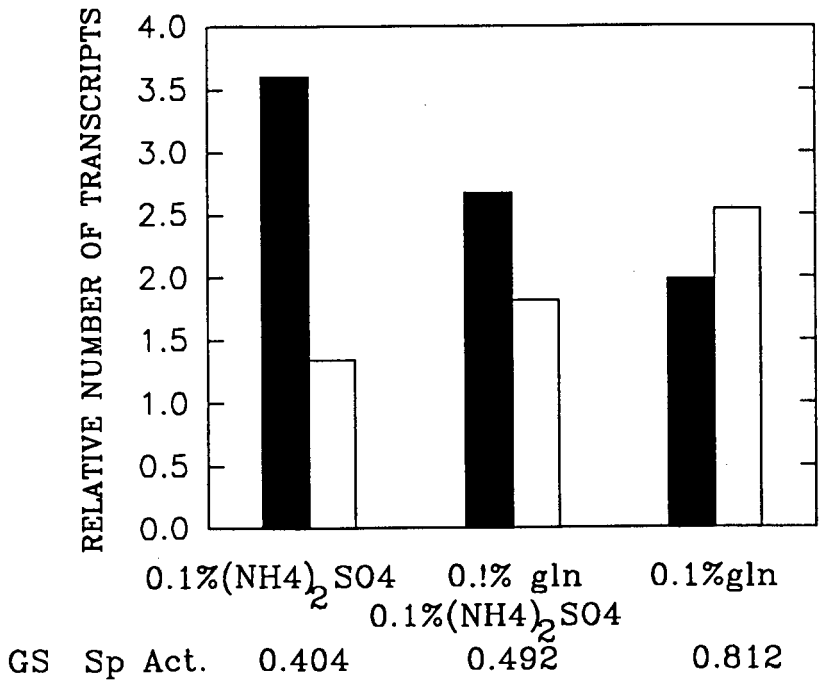
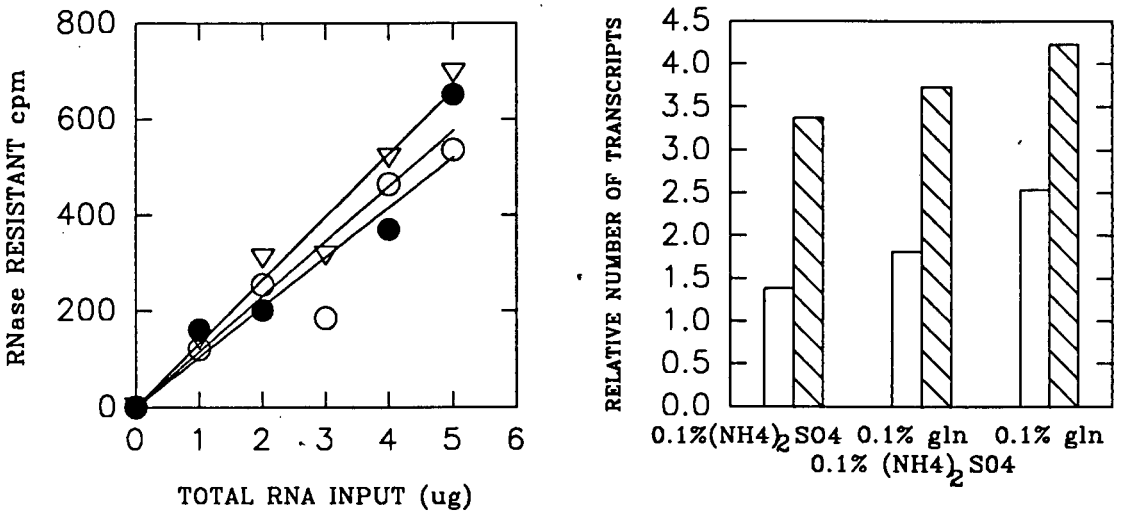


Fig. 3.6. Levels of *glnA* AS RNA, *glnA* mRNA, putative *glt S* mRNA and GS activity in *C. acetobutylicum* cells grown under N-limiting (GSMM-0.1%gln), N-rich (GSMM-0.1%gln-0.1%$(NH_4)_2SO_4$) and N-rich (GSMM-0.1%$(NH_4)_2SO_4$) conditions. **(a)** Levels of AS RNA and mRNA. Total RNA was prepared from cells grown in three cultures for

72h under the various N conditions and hybridized to a single stranded RNA probe generated from pSPAS, pSPgln700 or pglt78. The quantity for the RNA-RNA hybrid for the *glnA* AS RNA (solid circles), *glnA* mRNA (open triangles), or the putative *gltS* mRNA (not shown) was assayed in each sample after digestion with RNaseA and RNaseT. Data are presented as RNase-resistant counts per min versus total RNA input. Each value represents the average of three determinations. **(b)** Relative number of of *glnA* AS RNA and *glnA* mRNA transcripts, and GS specific activity in cells harvested at 72h from the three N conditions, respectively. The slopes of the lines (shown in panel a) were used to calculate the relative number of *glnA* AS RNA transcripts (solid bars) and *glnA* mRNA (open bars) transcripts under the three specified N conditions. GS specific aactivity was determined and is expressed as micromoles of γ-glutamyl hydroxymate per milligram of protein per min; each value represents the average of three determinations.

c.



(c) Relative number of *glnA* mRNA and putative *gltS* mRNA transcripts in the same *C. acetobutylicum* cells, grown for 72h under the three specified N conditions. The quantity for the RNA-RNA hybrid for the putative *gltS* mRNA from *C. acetobutylicum* cells grown in GSMM-0.1%gln (open triangles), GSMM-0.1%gln-0.1%$(NH_4)_2S0_4$ (open circles) and in GSMM-0.1%$(NH_4)_2SO_4$ (solid circles) is shown in the graph on the left. The slopes of the lines shown in this graph were used to calculate the relative number of the putative *gltS* mRNA transcripts (cross-hatched bars) shown in the bar graph on the right. The relative number of *glnA* mRNA transcripts corresponds to the data shown in (b) (open bars).

3.3.6. Analysis of the transcription levels of the putative *C. acetobutylicum gltS* mRNA encoding the GOGAT small subunit. A molecular titration assay based in the RNase protection assay was performed with total RNA isolated from *C. acetobutylicum* cells grown for 72 h in N-limiting (GSMM-0.1%gln) medium, N-rich (GSMM-0.1% gln-0.1% $(NH_4)_2SO_4$) and N-rich (GSMM-0.1% $(NH_4)_2SO_4$) media respectively. The transcription prevalence levels of the putative *gltS* mRNA are shown next to the prevalence levels of the *glnA* mRNA in Fig 3.6 (c). Higher putative *gltS* mRNA levels in comparison to *glnA* mRNA levels were detected in *C. acetobutylicum* cells grown under N-rich and N-limiting conditions. However, the highest difference was detected in cells grown in N-rich (GSMM-0.1% $(NH_4)_2SO_4$) media. In this medium the putative *gltS* mRNA levels were 2.44-fold higher than *glnA* mRNA levels. In cells grown in N-limiting (GSMM-0.1%gln) media the least difference was detected, the *gltS* mRNA levels were 1.66-fold higher than the *glnA* mRNA levels. Although slight differences in the *gltS* mRNA levels were detected in *C. acetobutylicum* cells grown in N-limiting medium compared to the same cells grown in N-rich media, these differences were not as great as the variation between the *glnA* mRNA levels determined in the same cells. In *E. coli* cells, GOGAT is as well derepressed by growth in a N limiting medium (i.e. gln as a N source) as it is in a N-rich medium (i.e. ammonia as a N source), and this enzyme is repressed in medium supplemented with glu (Castaño et al., 1988). GOGAT synthesis has been reported to be derepressed in *B. subtilis* cells by growth in N-rich conditions (supplemented with ammonia) and markedly repressed when the same cells where grown in medium supplemented with glu (Pan and Coote, 1979). In this study, no significant repression was observed in *B. subtilis* cells by growth in medium supplemented with gln.

3.3.7. CONCLUSION

Previous work on the molecular analysis of the *C. acetobutylicum glnA* region described the presence of a putative AS RNA linked to a P_3 promoter sequence. DNA hybridization analysis revealed that the *glnA* AS RNA coding sequence was only present in the DNA region downstream of the *C. acetobutylicum glnA* gene within the *C. acetobutylicum* chromosome and that this *glnA* AS RNA coding sequence presents high homology with the 5'start region of the *glnA* gene.

Analysis of the *glnA* gene and *glnA* AS RNA expression was performed in the host bacterium *C. acetobutylicum*. A defined minimal media including N-rich and N-limiting conditions for growth of *C. acetobutylicum* cells was developed. Expression of the predicted *glnA* AS RNA of 43 bases was demonstrated in *C. acetobutylicum* cells. In these cells, the expression of the *glnA* AS RNA was differentially regulated relative to the regulation of *glnA* mRNA and GS activity by N conditions. High expression levels of *glnA* AS RNA and low *glnA* mRNA and GS activity levels were detected in cells grown under N-rich conditions and the opposite was detected in *C. acetobutylicum* cells grown under N-limiting conditions. A comparison between the levels of *glnA* mRNA and the putative *gltS* mRNA levels (encoding for the putative small subunit of *C. acetobutylicum* GOGAT enzyme), was performed in *C. acetobutylicum* cells grown under N-rich and N-limiting conditions. Although higher putative *gltS* mRNA than *glnA* mRNA levels were detected under either high or low N conditions, a slight difference was detected in the putative *gltS* mRNA levels in the cells grown for 72 h under the three N conditions.

CHAPTER FOUR

INVESTIGATION OF ADDITIONAL REGULATORY ELEMENTS INVOLVED IN *C. ACETOBUTYLICUM glnA* GENE REGULATION.

SUMMARY: Since differential regulation by N levels of the *glnA* AS RNA expression relative to the *glnA* gene was demonstrated in *C. acetobutylicum,* additional regulatory element(s) affecting the *C. acetobutylicum glnA* system were investigated. *C. acetobutylicum* gene libraries were cotransformed *in trans* with an in-frame *glnA-lacZ* fusion construct for the screening of regulatory gene(s). Approximately 10,000 colonies carrying both the *glnA-lacZ* fusion construct and plasmids from the *C. acetobutylicum* gene libraries were tested for β-galactosidase expression. No alteration of the *lacZ* gene expression was detected in the cotransformed clones. However, DNA sequencing of the region situated downstream of the *C. acetobutylicum glnA* gene revealed the presence of an ORF located 199 to 766 bp from the 3' end of the *glnA* structural gene. The *glnA* AS coding region is located on the putative ribosome binding site and the 5'region of this *C. acetobutylicum* ORF. The protein encoded by this ORF showed 30% similarity with the carboxy terminus of the *Pseudomonas aeruginosa* aliphatic amidase regulator encoded by the *amiR* gene, which is involved in the positive regulation of the *amiE* gene. The amino terminus of this protein also has 28% and 26% similarity with the amino terminal region of the DegU and CheB response regulators, respectively. These regulators belong to the family of the response regulators involving two component signal transduction systems.

4.1. INTRODUCTION

The regulation of *glnA* gene expression has involved regulatory proteins in several prokaryote systems, including the enterobacteria and the Gram-positive sporeformer *B. subtilis* (Chapter 1). The participation of these protein regulators in signal transduction mechanisms in response to environmental sensory systems has also been demonstrated (Chapter 1). Furthermore, the response regulator as part of the "two component signaling system" is responsible for mediating changes in the *glnA* gene expression in response to sensor signals. In enterobacteria the sensor protein is represented by NtrB (NR_{II}) and the response regulator by NtrC (NR_I). Phosphorylation of NtrC stimulates *glnA* transcription from the promoter sequence *glnAp2*.

Another well studied signal transduction prokaryote system is the outer membrane (OmpC and OmpF) protein expression. In this system EnvZ represents the sensor protein and OmpR the regulator, both acting in response to external osmolarity changes. However, in addition to the action of the OmpR response regulator in this system, control of *ompF* gene expression by *micF* AS RNA illustrates an alternative regulatory mechanism (Chapter 1).

The implications for the control of the expression of the C. *acetobutylicum glnA* gene by the *glnA* AS RNA have not excluded the involvement of other regulatory element(s). In the Gram-positive *B. subtilis* strain the gene coding for the GlnR regulator was located in the DNA region situated between the *glnA* promoter and the 5'end of the structural *glnA* gene (Chapter 1). DNA sequencing of the region situated upstream of the C. *acetobutylicum glnA* gene did not reveal the presence of an open reading frame(s) coding for a regulatory gene(s) (Paul Janssen, PhD thesis). However, several direct repeats that have been proposed to be binding sites for regulatory proteins belonging to the *LysR* family were found to be located in the DNA region upstream of the C.

acetobutylicum glnA gene (Paul Janssen, PhD thesis). These direct repeats have a high degree of similarity to a 9 bp DNA sequence with consensus 5'-ATATTGTTT-3' which is present in a number of target DNA sequences of genes coding for enzymes involved in aa biosynthesis. In addition, the DNA region situated upstream of the *glnA* gene was searched for representative operator sequences used by proteins containing the α-helix-turn-α helix structure (TNTNAN-2-5N-NTNANA) (Weickert and Chambliss, 1990). One of these operator sequences was found to be present 136 nt upstream from the promoter sequence P_1. This sequence is very similar to the operator sequences upstream of *B. subtilis glnRA* and *amyR*, *B. cereus glnA*, and *E. coli lac* and *gal* genes (TGTNAN-2-5N-NTNACA) (Nakano et al., 1989; Schreier et al., 1991). These operator sequences and the differential expression of the *glnA* AS RNA (Fierro-Monti et al., 1992) suggest the participation of additional regulatory mechanisms involving the expression of other gene(s). The product(s) of the gene(s) could be implicated in the regulation of the *glnA* AS RNA and *glnA* gene expression. Therefore, it was interesting to investigate the participation of the gene(s) involved in the regulation of *C. acetobutylicum glnA* gene expression. The studies done on the search for these genes are described in this chapter.

4.2. MATERIALS AND METHODS

4.2.1. Bacterial strains and growth conditions. *E. coli* strains (Appendix B.2) were grown as described in Chapter 2, unless otherwise stated in the text. *E. coli* JM109 served as a recipient for the Bluescript-SK plasmid derivatives, pHH730 and pHEV200 (used as nucleotide sequencing DNA templates) for the plasmid pupgln1300, and for two *C. acetobutylicum* chromosomal DNA libraries; one made in the pEcoR251 plasmid and the other in the *B. subtilis-E. coli* shuttle vector pEB1.

4.2.2. Preparation of nucleic acid. Plasmid DNA was prepared by the method of Ish-Horowicz and Burke, 1981 (Appendix A.1.1-2).

4.2.3. Plasmid constructions. Plasmid pupgln1300 contained a frame fusion of the 5'start of the *C. acetobutylicum glnA* gene and a 1.3kb DNA region situated upstream of this gene, subcloned upstream of the *lacZ* gene into a *SalI* site located within the tetracycline resistance gene of plasmid pACYC184 (Fig. 4.1). Plasmid pACYC184 carries the origin of replication from plasmid p15A (Chang and Cohen, 1978), which enables it to co-exist with the pBR322 replicons in vectors such as pEcoR251 or pEB1.

Plasmid pHH730 contained a 730 nt DNA fragment from the the 3'end and the region immediately downstream of *C. acetobtuylicum glnA* gene and plasmid pHEV200 included a 200 nt DNA insert from the region downstream of the *C. acetobutylicum glnA* gene. Sub-cloning strategies utilized were essentially performed according to the standard recombinant DNA techniques used by Maniatis et al., 1984. Nucleotide sequences of plasmid constructs were confirmed by sequence analysis as described in section 4.2.5.

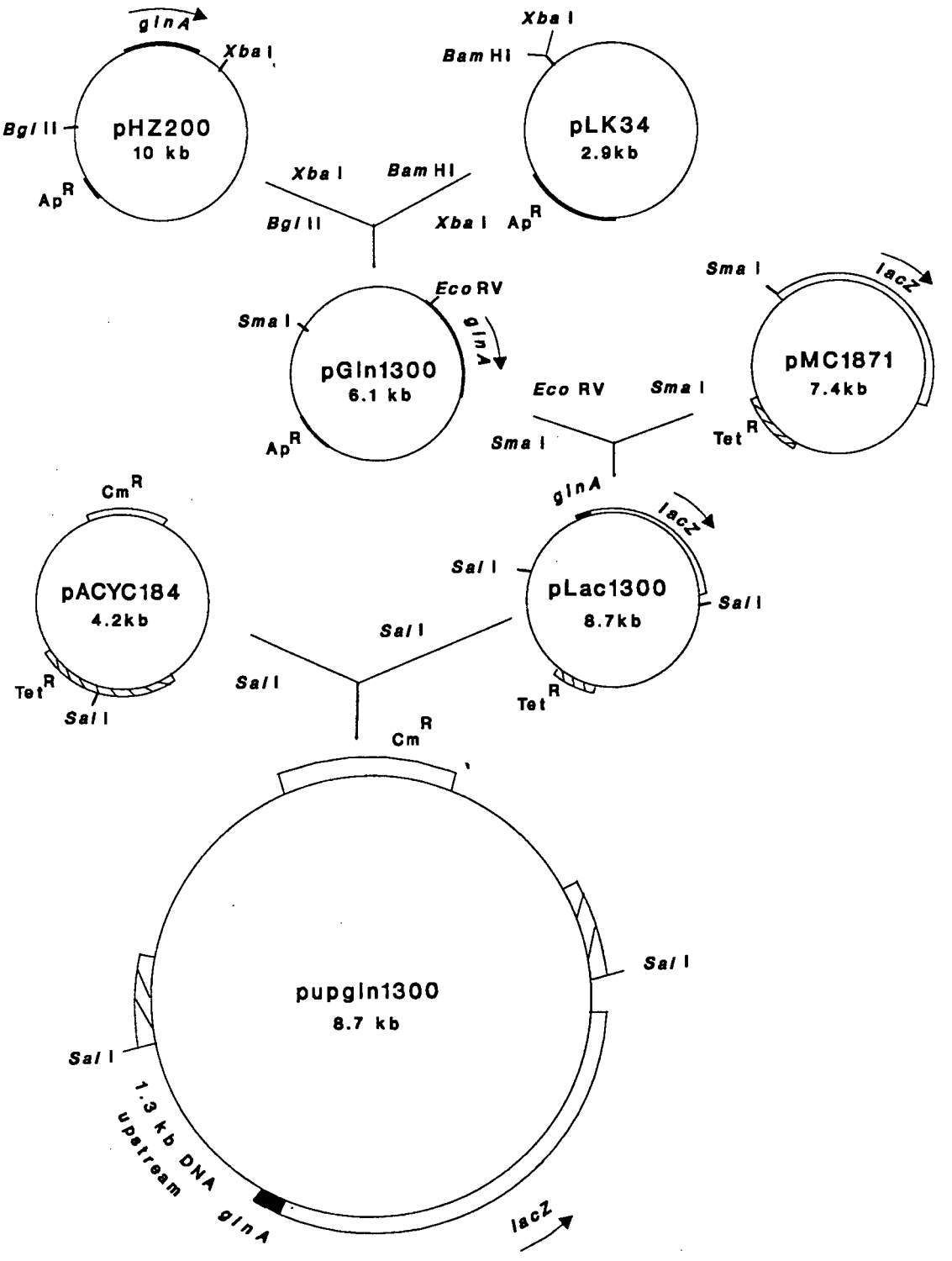


Fig. 4.1. Cloning strategy for the *glnA-lacZ* fusion vector pupgln1300. Plasmid pHZ200 was the original source of the C. *acetobutylicum glnA* region. Genes encoding for antibiotic resistance are abreviated as Ap (ampicillin), Tet (tetracycline), and Cm (chloramphenicol).

4.2.4. Screening for a *C. acetobutylicum glnA* regulatory gene by use of *C. acetobutylicum* gene libraries *in trans* with a *glnA-lacZ* fusion construct in *E. coli* JM109. In an attempt to identify a regulatory gene from a *C. acetobutylicum* gene library (made in pEcoR251) which interacted with the upstream region of the *glnA* gene, plasmid pugln1300 and the *C. acetobutylicum* gene library were cotransformed into the *E. coli* strain JM109. The expression mixes were plated onto LB medium supplemented with chloramphenicol, ampicillin and X-gal. As a control plasmid pCEcoR, a pEcoR251 derivative containing a small fragment of DNA subcloned into the *Bgl*II site of this plasmid and the plasmid pupgln1300 were used to cotransform *E. coli* JM109. Selection was done by monitoring the growth and blue/white colour of the transformants on the respective medium. Plasmid pugln1300 and the *C. acetobutylicum* gene library made in the *E. coli-B. subtilis* shuttle vector pEB1 were also used to cotransform the *E. coli* strain JM109. Plasmids pCEcoR and pupgln1300 were used to cotransform *E. coli* JM109 as a control.

4.2.5. Nucleotide sequencing. The nucleotide sequence of both strands of the DNA downstream of the *C. acetobtuylicum glnA* gene was determined using the contiguous DNA fragments subcloned into plasmid Bluescript-SK (plasmids pHH730 and pHEV200). DNA sequencing was carried out by the dideoxynucleotide triphosphate chain termination method described in Appendix 1.11.1-3.

The DNA and deduced aa sequences were analyzed on a IBM XT computer using the Genetic Computer Group Incorporated Sequence Analysis Software Package version 6.1 (GCG Package). All the current databases accompanying the GCG Package were screened for related nt and aa sequences.

4.3. RESULTS AND DISCUSSION

4.3.1. Attempts to clone a *C. acetobutylicum glnA* regulatory gene by screening *C. acetobutylicum* gene libraries *in trans* with a *glnA-lacZ* fusion construct in the *E. coli* strain JM109. The *C. acetobutylicum* chromosomal gene library made in the plasmid pEcoR251 and the *glnA-lacZ* fusion plasmid pupgln1300 were utilized to cotransform the *E. coli* strain JM109. The fusion plasmid pupgln1300 contained the 5' start of the *glnA* region and 1.3kb DNA upstream of this gene subcloned upstream of the *lacZ* gene. Expression of the *glnA* regulatory gene(s) present in the *C. acetobutylicum* gene library could alter the expression of β-galactosidase by interacting with the *glnA* upstream promoter region. Approximately 10,000 transformants carrying both the upstream *glnA-lacZ* fusion and at least one plasmid (48 randomly chosen clones were confirmed by miniprep DNA restriction analysis in agarose electrophoresis) from the gene library were screened. No significant increase or decrease in β-galactosidase expression was detected in the colonies analyzed.

The same approach was used in further attempts to clone a *glnA* regulator gene. A *C. acetobutylicum* chromosomal DNA library made in a *B. subtilis-E. coli* shuttle vector pEB1 and the *glnA-lacZ* fusion plasmid pupgln1300 were to cotransform the *E. coli* strain JM109. Approximately 10,000 colonies carrying both the *glnA-lacZ* fusion plasmid pupgln1300 and a plasmid from the new *C. acetobutylicum* gene library were tested for β-galactosidase expression. No particular alteration of the *lacZ* gene expression was detected in the clones. The use of this screening system in the *E. coli* strain JM109 was unsuccessful in isolating a *glnA* regulatory gene(s).

4.3.2. Nucleotide sequence of the region downstream of the *C. acetobutylicum glnA* gene. Nucleotide sequencing was used as another strategy to reveal the presence of regulatory gene(s) that could be affecting the expression of the *C. acetobutylicum glnA* gene. Although there was no phenotypic evidence of other elements involved in *glnA* regulation other than the AS RNA coding sequence situated downstream of the *C. acetobutylicum glnA* gene, it was of interest to investigate the genetic determinant(s) present in this DNA region. Two plasmids (pHH730 and pHEV200) containing contiguous DNA fragments from the region located downstream the *glnA* gene were sequenced in both directions. The region of overlap with the 3' end of the *glnA* gene was identified in plasmid pHH730. One ORF was present downstream of the *glnA* gene on the same DNA strand (Fig. 4.2 and Fig. 4.3).

This ORF was in the same orientation with respect to the *glnA* gene. However, the *glnA* AS RNA coding sequence was situated on the opposite DNA strand in the 5'start region of this putative gene. The *glnA* AS RNA was therefore complementary to the ribosome binding site and the first 8 codons of the ORF mRNA. From the presumptive ATG start codon to the presumptive TAA stop codon this ORF contained 566 bp, which encoded a polypeptide of 189 aa residues (Fig. 4.3). The M_r of the predicted polypeptide was 21,400 kd. The ATG start codon was not preceded by a classical ribosome-binding site (Shine and Dalgarno, 1974). An AGGCGGGGA was located 14 bp upstream of the ATG start codon (Fig. 4.3). A putative -10 and -35 region was separated by 16 bp.

On the basis of identical aa sequence similarities, the translated aa sequence showed 30% similarity with the carboxy terminal domain (18 identical residues from a sequence of 60 residues) of the alifatic amidase regulator (AmiR) of *P. aeruginosa*.

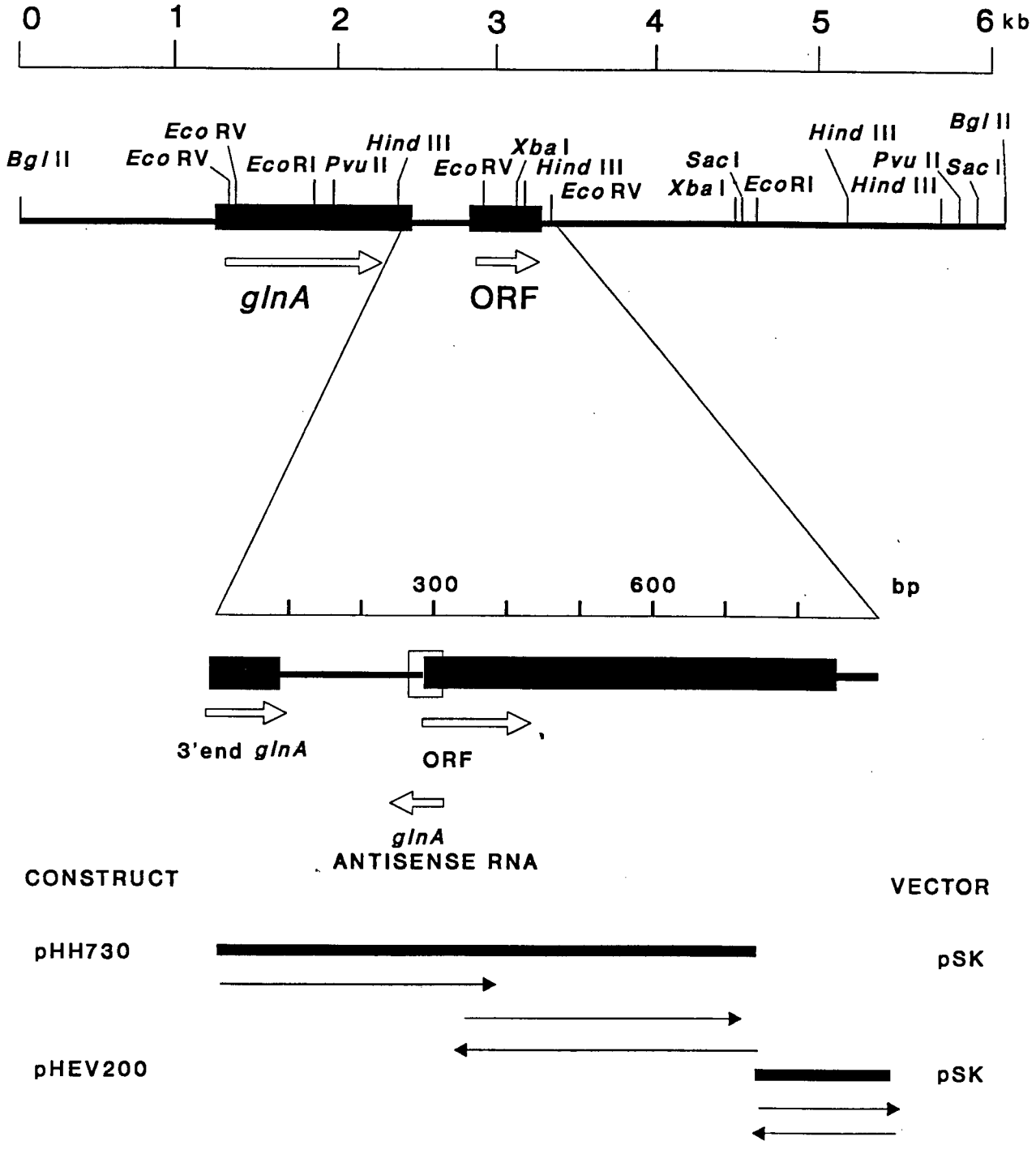


Fig. 4.2. Restriction map of the C. *acetobutylicum glnA* region, subclones derived from the region downstream of the *glnA* gene and the sequencing strategy for the ORF located in this region. The bold lines, single thin line and open arrows represent the C. *acetobutylicum* insert DNA, the DNA sequences previously reported (Janssen et al., 1989) and the ORF, respectively. The thin arrows represent the extent and the direction of sequencing of the DNA templates.

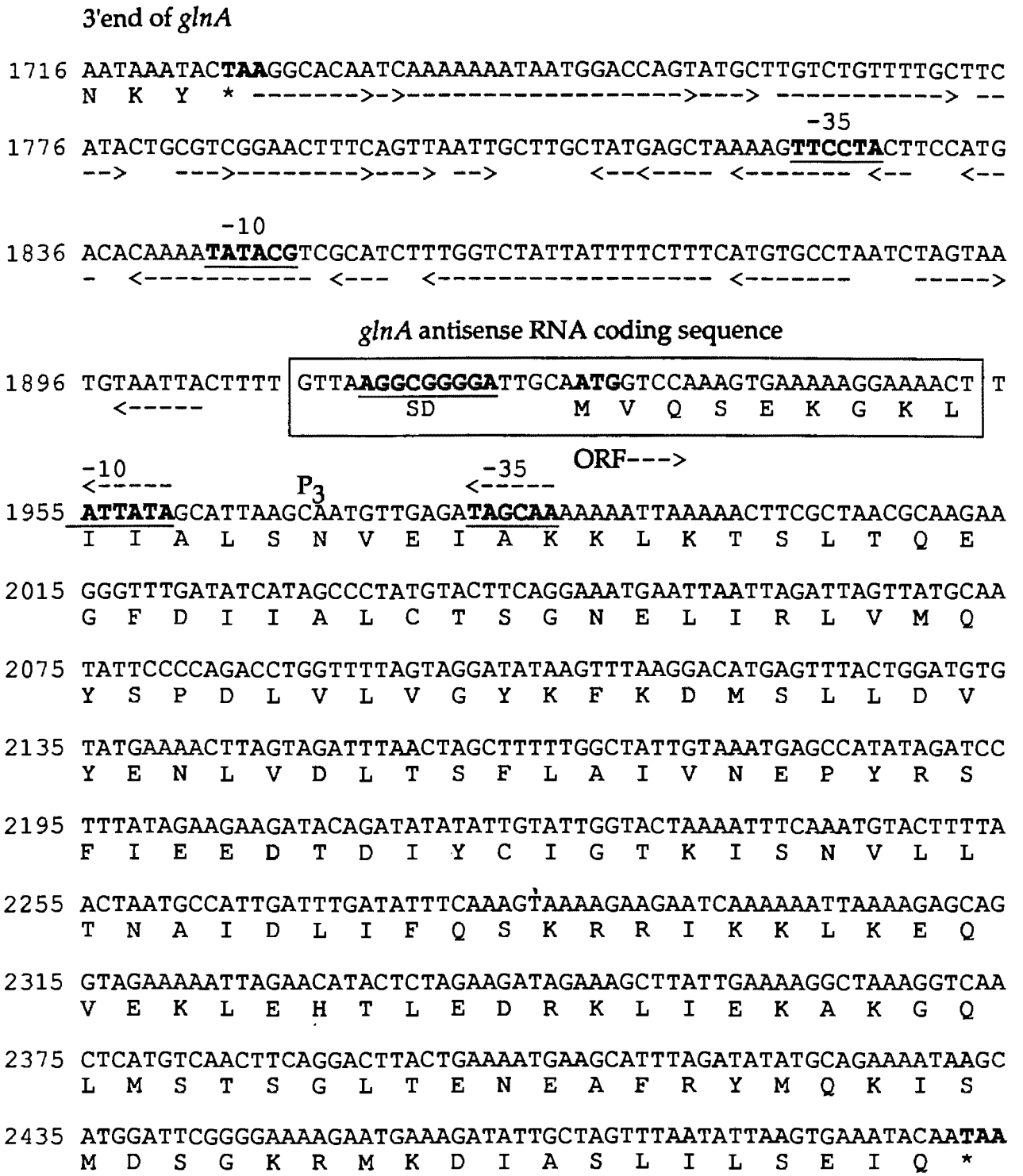


Fig. 4.3. Nucleotide sequence downstream of the *C. acetobutylicum glnA* gene containing the ORF of the putative regulatory gene. The deduced aa sequences are shown in single-letter code below the coding sequences. The nucleotide sequence is numbered throughout. The boxed sequence represents the *glnA* antisense RNA coding sequence. The arrows indicate the inverted repeat sequences and the orientation of the transcription of the antisense RNA or the ORF, respectively. The putative promoter (-35 and -10 region) and ribosome-binding site (SD) are in boldface type and underlined. The start codon and the end of the sequence are indicated in boldface type and with an asterix below the codon.

A comparison between the carboxy terminal region from both aa sequences is shown in Fig. 4.4a. The deduced aa sequence of the ORF showed twenty eight percent (13 identical residues from a sequence of 46 residues) and twenty percent (13 identical residues from a sequence of 62 residues) similarity with the amino terminal sequences of the *B. subtilis* DegU and CheB response regulators, respectively. A comparison of the amino terminal region of the translated aa sequence of this ORF was made with the conserved amino terminal region of other response regulators which are members of the family of two component signal transduction systems (Fig. 4.4b). This conserved amino terminal region represents the regulatory region from the response regulator family (Chapter 1).

DNA sequencing of the region downstream of the *P. aeruginosa amiE* gene has identified the presence of two regulatory genes *amiC* and *amiR*. The *amiR*-encoded alifatic amidase regulator is involved in the positive control of the transcription of the amidase encoded *amiE* gene via an antitermination mechanism (Cousens et al., 1987; Drew and Lowe, 1989, Wilson and Drew, 1991). A rho independent transcription terminator is present in the region between the *amiE* promoter and the *amiE* structural gene. The *amiR* gene has been suggested to act by allowing RNA polymerase to read through the rho-independent terminator (Drew and Lowe, 1989). The *amiC* gene encodes for the negative regulator AmiC, which down regulates the expression of the amidase enzyme. Transcription of the *amiC* and the *amiR* genes generate a single mRNA transcript (Wilson, 1991). AmiC has been demonstrated to be a specific amide binding protein which migh act inhibiting AmiR activity by forming an AmiC-AmiR complex.

```
ORF   125  KLK EQV E K L EHTLEDRKL I EK AK GQ LM STS G LT 158
AmiR  131  KLK QKT E Q L QDRIAGQAR I NQ AK VL LM QRH G WD 164

ORF   159  E N EA FRYMQKIS M DSGKRMKD IA SLI L SEIQ
AmiR  165  E R EA HQHLSREA M KRREPILK IA QEL L LGNEPSA
```

Fig. 4.4a. Comparison of the carboxy terminal aminoacid sequence of the *C. acetobutylicum* putative regulator (ORF) with the alifatic amidase regulator from *P. aeruginosa* (Lowe et al., 1989). The amino acids are identified by the single letter code and identical residues are boxed. The numbers preceding the amino acid sequences indicate the position of the residues.

```
ORF    MVQSEKG KLII ALSNVE I AKK LK TS L T-QEGFDII A LCTS G
cheB   MA---H- RILI VDDAAF M RMM IK DI L -VKNGFEVV A EAEN G
degU   ---MTKV NIVI IDDHQL F REG VK RI L DFEPTFEVV A EGDD G
ntrC   ---MQRG IAWI VDDDSS I RWV LE RA L TGAGLSCT- - TFES G
spoOA  ---MEKI KVCV ADDNRE L VSL LS EY I EGQEDMEVI G VAYN G
spoOF  ---MMNE KILI VDDQYG I RIL LN EV F NKEGYQTF- - QAAN G
sfrA   ---MQTP HILI VEDELV T RNT LK SI F EAEGYDVF- - EATD G
cheY    MADKEL KFLV VDDFST M RRI VR NL L KELGFNNVE - EAED G
ompR   --MQEMY KNLV VDDDMR L RAL LE RY L TEQGFQVRS V ANAE Q
phoB   ----MAR RILV VEDEAP I REM VC FV L EQNGFQPVE A EDYD S

ORF      N E LIRLVMQYS PDLV LVGYKFKD MSLL DVYEN LV DLTSFLA I
cheB     A Q AVEKYKEHS PDLV TMDITMPE MDGI TALKE IK QIDAQAR I
degU     D E AARIVEHYH PDVV IMDINMPN VNGV EATKQ LV --ELYPE S
ntrC     N E VLDALTTKT PDVL LSDIRMPG MDGL ALLKQ IK --QRHPM L
spoOA    Q E CLSLFKEKD PDVL VLDIIMPH LDGL AVLER LR ESDLKKQ P
spoOF    L Q ALDIVTKER PDLV LLDMKIPG MDGI EILKR MK --VIDEN I
sfrA     A E MHQILSEYD INLV IMDINLPG KNGL LLARE LR ---EQAN V
cheY     V D ALNKLQAGG YGFV ISDWNMPN MDGL ELLKT IR ADGAMSA L
ompR     M D RLLTRESFH LMVL --DLMLPG EDGL SICRR LR --SQSNP M
phoB     A V NQLNEPWPD LILL --DWMLPG GSGI QFIKH LK RESMTRD I
```

Fig.4.4b. Comparison of the amino terminal amino acid sequence of the putative regulator (ORF) with some of the response regulators from the family of two components signal transduction systems. The amino acids are identified by the single letter code and similar sequences are boxed. Residues that are identical in at least four of the ten aligned proteins are boxed. The references for the amino acid sequences of the proteins included in this comparison are as follows: *cheB* (Bischoff and Ordal, 1991), *degU* (Henner et al., 1988), *ntrC* (Buikema et al., 1985), *spoOA* (Ferrari et al., 1985), *spoOF* (Trach et al., 1985), *sfrA* (Drury and Buxton, 1985), *cheY* (Mutoh and Simon, 1986), *ompR* (Wurtzel et al., 1982) and *phoB* (Makino et al., 1986).

The inhibitory effect of AmiC on AmiR depends on the presence or absence of amides (Wilson et al., 1993).

Two other bacterial transcription antitermination systems have been demonstrated to be acting on the *bgl* operon in *E. coli*, and the *sac* operon of *B. subtilis* (Schnetz and Rak, 1988; Le Cok et al., 1989). A membrane bound protein is involved in down regulating the antiterminator activity in both of these systems. These proteins, BglF and SacX are involved in a phosphotransfer system which depends on phosphoenolpyruvate (Le Coq et al., 1989).

The sensor protein DegS in association with the response regulator DegU participate in the transcriptional activation of genes encoding degradative enzymes (Henner et al., 1988). The two component CheA and CheB proteins are involved in signal transduction of chemotaxis. The CheB amino terminal domain has a regulatory function. The carboxy terminal domain involves a methylesterase activity. CheB increases its methylesterase activity when its regulatory domain is phosphorylated by the sensor protein kinase CheA (Stewart, 1992).

Therefore, based on the similarity with other protein sequences there is a possibility that the *C. acetobutylicum* ORF could play a role as a response regulator protein, and also that it might be involved in the positive control of transcription or antitermination. An inverted repeat sequence situated upstream of *C. acetobutylicum glnA* gene overlaps the DNA sequence of the promoter P_2. Transcription from the promoter P_1 region, which precedes the inverted repeat could form a stem loop structure (ΔG=-11 kcalmol^{-1}) (Fig. 4.5) and act as a factor independent transcription terminator (P. Janssen, PhD thesis).

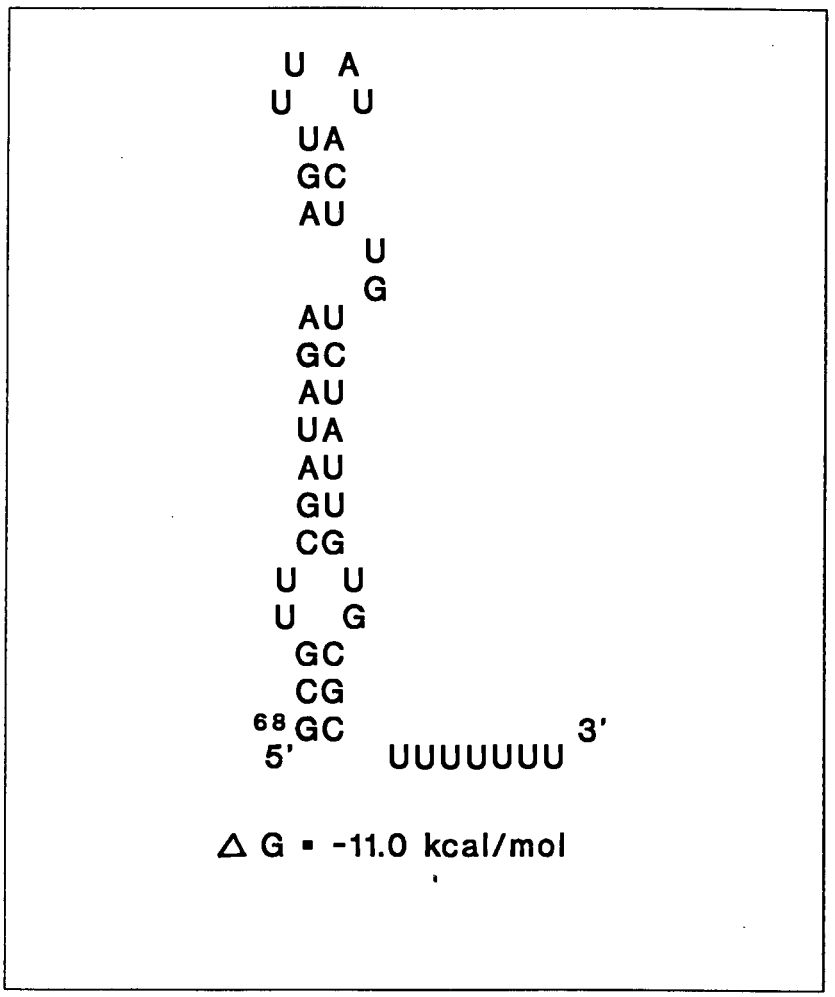


Fig. 4.5. Predicted secondary structure of the stem loop RNA transcribed from the promoter sequence P_1. The inverted repeat sequence encoding for this factor-independent transcription terminator overlaps the promoter sequence P_2, and is situated in the region upstream of the *C. acetobutylicum glnA* gene. The position within the *glnA* upstream sequence is indicated at the 5'end.

The *glnA* AS RNA coding region was present at the 5'start of the ORF. *glnA* AS RNA transcribed from the P_3 promoter sequence in the opposite orientation overlaps the ribosome binding site of the putative ORF mRNA. Expression of this AS RNA could then represent a fine-tuning regulatory system that affects not only the *glnA* gene expression, but also the expression of the putative response regulator gene. It will be important to analyze whether this putative response regulator affects the expression of the *glnA* gene. To prove this hypothesis further experiments have to be performed. These will involve *in vivo* and *in vitro* translation, purification of the regulatory protein, DNA binding studies and regulatory studies.

4.3.3. CONCLUSION

The presence of gene(s) or gene product(s) involved in the regulation of the expression of *C. acetobutylicum glnA* gene was investigated. The strategy used for the screening of two *C. acetobutylicum* gene libraries *in trans* with an in frame fusion *glnA-lacZ* plasmid was not successful. However, DNA sequencing of the region situated downstream of the *glnA* gene revealed an ORF encoding a putative polypeptide of 189 aa residues. On the base of identical aa, this ORF has 30% similarity with the carboxy terminal region of the *P. aeruginosa* alifatic amidase regulator, which is involved in the transcriptional regulation of the *amiE* gene via an antitermination mechanism. In addition, this ORF shows 28% and 26% similarity with the amino terminal region of the DegU and CheB response regulator proteins, respectively. The DegU and CheB proteins belong to the family of response regulators from the two component signal transduction systems.

Interestingly the *glnA* AS coding sequence overlaps the putative ribosome binding sequence and the 5'start of this *C. acetobutylicum* ORF, but would be transcribed in the opposite orientation from promoter P_3.

CHAPTER FIVE

GENERAL CONCLUSION

Prior studies involved the cloning of the *C. acetobutylicum glnA* region in *E. coli* as a host, (Usdin et al., 1988) followed by the DNA sequencing of the entire *glnA* region including the structural *glnA* gene and the DNA regions located upstream and downstream of this gene (Janssen et al., 1989). The presence of a putative AS RNA coding region with homology to the ribosome binding site and the 5'start of the *glnA* gene was revealed in the DNA region downstream of this gene (Janssen et al., 1989). The aim of this study was to advance the knowledge on the regulation of N metabolism in *C. acetobutylicum* by investigating *glnA* gene expression with special emphasis on the regulatory mechanism(s) involved.

Initially, the expression of the *C. acetobutylicum glnA* gene was analyzed in *E. coli* to investigate the role of any *C. acetobutylicum* regulatory gene(s) in this heterologous host. Minimal media including N-rich or N-limiting conditions that supported good growth of the *E. coli* cells carrying the *C. acetobutylicum glnA* region were developed. Expression of *C. acetobutylicum* GS activity in these cells showed only slight differences when compared under the respective N conditions in which the *E. coli* strains were grown. However, GS activity levels in *E. coli* cells transformed by a plasmid containing the entire *glnA* region compared to the same cells transformed by a construct in which the AS RNA coding region was deleted resulted in decreased levels of GS activity in the cells containing the AS RNA region. RNA protection analysis in *E. coli* cells carrying a plasmid which included the AS coding sequence, demonstrated the presence of the *glnA* AS RNA transcript (Fierro-Monti et al., 1992). Twenty five percent inhibition of GS activity by the *glnA* AS RNA was demonstrated when *E. coli* cells grown in N-

limiting medium were cotransformed by a low copy number plasmid carrying the *glnA* region and a high copy number plasmid providing additional copies of the AS RNA coding sequence. The *glnA* AS RNA was therefore expressed efficiently in *E. coli* and could act *in trans*.

Further analysis of the expression of the *glnA* gene and the *glnA* AS RNA expression was performed in the original host *C. acetobutylicum*. This analysis required the development of defined minimal media including N-limiting and N-rich conditions for *C. acetobutylicum* cell growth. Expression of the predicted *glnA* AS RNA of 43 bases was demonstrated in the original *C. acetobutylicum* host. The expression of GS activity and the *glnA* mRNA was regulated by N levels in *C. acetobutylicum* cells. Expression of the *glnA* AS RNA was differentially regulated by N levels when compared to GS activity and *glnA* mRNA production in the same *C. acetobutylicum* cells (Fierro-Monti et al., 1992). The utilization of a suitable gene transfer system to introduce genes back into the original *C. acetobutylicum* host and the development of the *C. acetobutylicum* mutant strains defective in either the *glnA* region (including the AS RNA coding region) would facilitate the further analysis of the regulatory mechanism(s) affecting *glnA* gene expression. Unfortunately attempts in this laboratory to specifically inactivate the *C. acetobutylicum glnA* gene by insertional mutagenesis utilizing a deleted *glnA* fragment containing an antibiotic resistant gene have not been succesful. There are no reports as yet of a recombination mutagenensis system in *C. acetobutylicum* and a major problem is the lack of an efficient antibiotic resistant marker for selection purposes.

The putative *gltS* gene coding for the small subunit of *C. acetobutylicum* GOGAT was cloned in a 1.63kb DNA region of the *C. acetobutylicum* chromosome (Helen Stutz, PhD thesis). As the GS and the GOGAT pathways are part of the synthesis of gln and glu, it was of interest to analyze the expression of this putative *gltS*

gene in *C. acetobutylicum*. When the *glnA* mRNA and the putative *gltS* mRNA levels were compared in *C. acetobutylicum* cells grown under N-rich and N-limiting conditions, higher levels of *gltS* mRNA were detected than *glnA* mRNA, and only slight differences of the *gltS* mRNA levels were observed in the cells grown under the different N conditions.

The differential regulation by N levels of the *glnA* AS RNA expression relative to the *glnA* gene in *C. acetobutylicum* cells, and the presence of putative regulator DNA binding sequences in the DNA region situated upstream of the *glnA* gene, (Fierro-Monti et al., 1992) suggested that other regulatory element(s) could be playing a role in the *C. acetobutylicum glnA* system. Therefore, the participation of additional gene(s) or gene product(s) involved in alternative regulatory mechanism(s) affecting this system was investigated. *C. acetobutylicum* gene libraries were cotransformed *in trans* with an in-frame fusion *glnA-lacZ* plasmid as a strategy for the screening of the regulatory gene(s). Although this method was not successful, DNA sequencing of the region located downstream of the *glnA* gene revealed the presence of an ORF coding for 189 aa. The *glnA* AS RNA coding region is located on the putative ribosome binding site and the 5'start of the ORF. This ORF showed 30% similarity with the carboxy-terminal region of the *P. aeruginosa* aliphatic amidase regulator encoded by the *amiR* gene, which is involved in the positive regulation of the *amiE* gene via a transcriptional antitermination mechanism. The *amiR* gene is linked to an upstream *amiC* gene coding for a negative regulator acting on the *amiE* gene. The *C. acetobutylicum* ORF has 28% and 26% similarity with the amino terminus of the DegU and CheB response regulators, respectively. These regulators have been classified as part of the family involving component signal transduction systems and have been shown to play a regulatory role in response to a sensor signal. The *C. acetobutylicum* ORF could represent a positive regulator which responds via signal transduction to the N concentration in the environment. The presence of

the *glnA* AS RNA coding region in the 5' start of this putative regulator could implicate a link between two inverse *C. acetobutylicum glnA* regulatory mechanisms (Fig. 5.1). A proposed model for the regulation of the *C. acetobutylicum glnA* gene expression could involve both, the activation of *glnA* transcription by the putative regulator and the inhibition of the *glnA* mRNA translation by the *glnA* AS RNA. Under low N conditions a signal transduction mechanism could induce the expression of the putative regulator. This putative regulator could interact with the factor independent terminator located upstream of the *glnA* gene allowing the RNA polymerase to read through and transcribe the *glnA* gene into *glnA* mRNA transcripts. Under rich N conditions a signal could be sensed by the promoter P_3, activating the expression of *glnA* AS RNA. The *glnA* AS RNA could reduce the expression of both, GS enzyme by binding to the 5'end of the *glnA* mRNA and the putative positive regulator by interacting with the 5'region of the mRNA encoding it.

Further experiments involving *in vivo* and *in vitro* translation, purification, DNA binding studies, and analysis of the regulation of the expression of *C. acetobutylicum* ORF will help to elucidate the functional properties of this putative regulator gene.

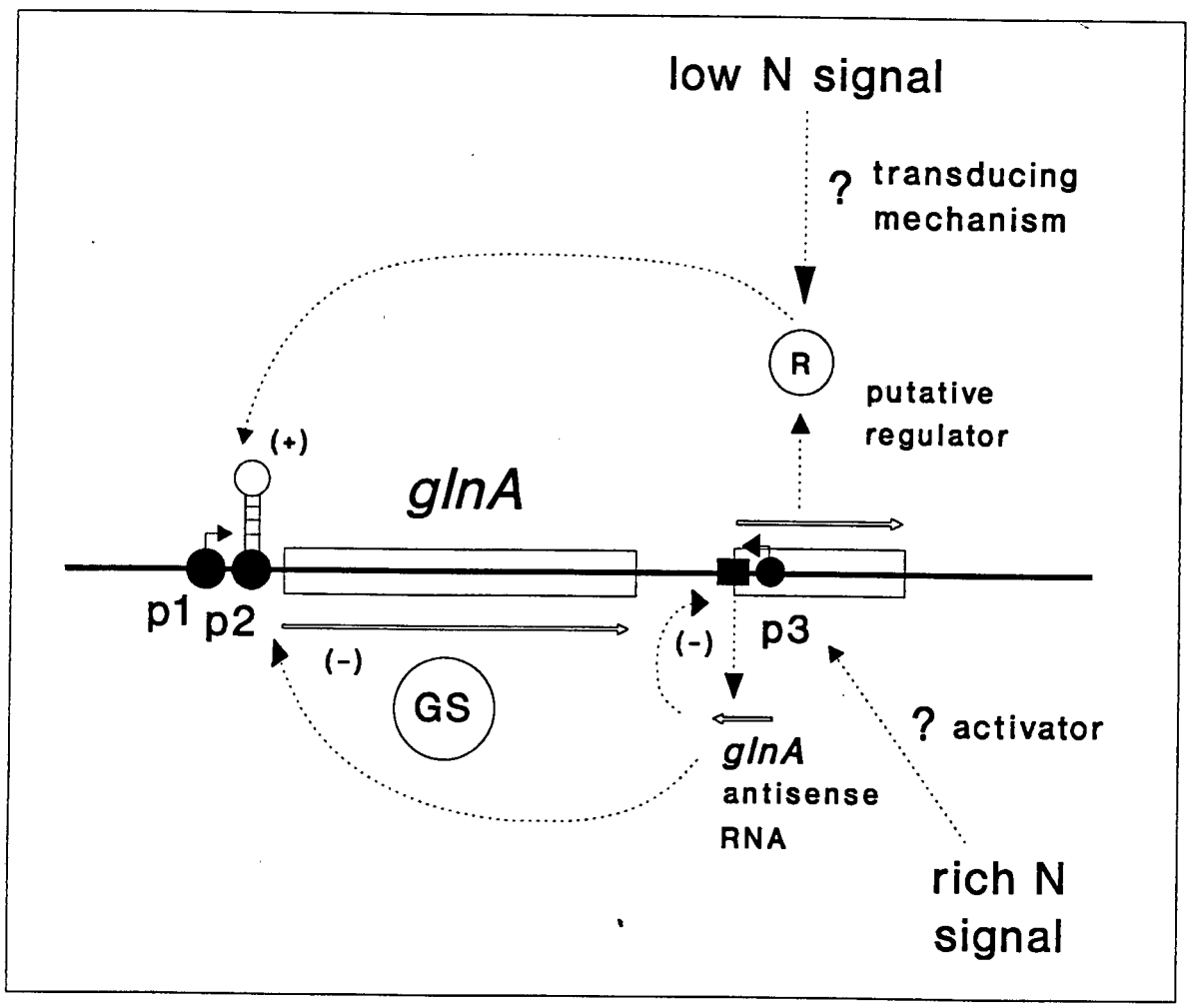


Fig. 5.1. Model for the regulation of the *C. acetobutylicum glnA* gene by N. The *glnA* region is characterized by the presence of the *glnA* structural gene, the *glnA* AS RNA region and the putative regulator gene located downstream of *glnA*. The promoter sequences P_1, P_2 and the rho independent terminator structure are located in the region upstream of *glnA*. The promoter sequence P_3 is situated in the 5' region of the putative regulator gene and directs the synthesis of the *glnA* AS RNA which has the potential to bind both, the 5'end of the *glnA* mRNA and the 5'end of the putative regulator mRNA transcripts, reducing expression of the respective genes in response to a rich N signal. The putative regulator could act as a transcriptional antiterminator by interacting with the rho independent terminator in response to a low N signal transduction mechanism.

APPENDIX A

STANDARD METHODS, MEDIA, BUFFERS AND SOLUTIONS

A.1 STANDARD METHODS

A.1.1 Small scale isolation of plasmid DNA (miniprep)

Plasmid DNA was isolated from a 5ml overnight culture (LB + Ap, 100 μg/ml) as described by Ish-Horowicz and Burke (1981). Cells from a 1.5 ml sample of the culture were harvested by centrifugation in an Eppendorf microfuge tube for 1 min. The pellet was resuspended in 200 μl Solution I (50mM glucose; 25mM Tris-HCl, pH 8.0), incubated for 5 min at room temperature, and then 400 μl of Solution II (0.2 M NaOH, 1% (w/v) SDS) was added. The sample was vortexed briefly and placed on ice for 5 min, before the addition of 300 μl ice-cold Solution III (5 M KOAc, pH 4.8). The sample was vortexed briefly, and, after 5 min on ice, cellular debris and denaturated chromosomal DNA were pelleted by centrifugation for 5 min. The supernatant (750 μl) was removed to a fresh tube, an equal volume of isopropanol was added and the DNA precipitated by centrifugation for 5 min. The DNA pellet was resuspended in TE (600μl) (Appendix A.2.7) before adding $NaClO_4$ (60 μl, 5 M) and an equal volume of isopropanol, and precipitating the DNA by centrifugation for 15 min. The DNA pellet was washed with 70% ethanol, air dried and resuspended in 50 μl TE buffer.

A.1.2 Large scale isolation of plasmid DNA (maxiprep)

A 200 ml culture was grown overnight at 37°C in the presence of the appropiate antibiotic. The cells were harvested by centrifugation at 6,000 x g for 5 min and

then resuspended in 4 ml Solution I. After 5 min at room temperature 8 ml Solution II was added, and the mixture was kept on ice for 5 min, before the addition of 6 ml ice cold Solution III. After a further 5 min on ice the cellular debris was removed by centrifugation at 12,000 x g for 10 min. An equal volume of isopropanol was added to the supernatant and the DNA was precipitated by centrifugation at 27,000 x g for 15 min. The pellet was washed with 70% ethanol and resuspended in 4.2 ml TE buffer, and purified by isopycnic CsCl-EtBr ultracentrifugation (Maniatis et al., 1982). The plasmid preparation was prepared for ultracentrifugation by the addition of CsCl (1 mg/ml) and EtBr (0.5 ml of a 10 mg/ml stock). The solution was centrifuged at 27 000 x g for 15 min to precipitate any remaining protein debris. The refractive index of the supernatant was adjusted to 1.396, the sample sealed in Beckman Quickseal ultracentrifuge tubes and centrifuged for 12 h at 55 000 rpm at 15°C in a Beckman Vti 65.2 rotor. The plasmid DNA band was visualized by long wave UV light (350 nm), and removed in the smallest volume possible. The EtBr was removed by extraction (3 times) with equal volumes of the addition of two volumes of water followed by an equal volume of isopropanol, and centrifugation in an Eppendorf microfuge for 15 min. The pellet was resuspended in 200 µl TE buffer and the concentration was determined spectrophotometrically by measuring the absorbance of 10 µl (diluted in TE) between 220 and 310 nm. The concentration was determined by using the relationship $A_{260} = 1$ for 50 µg/ml double-stranded DNA.

A.1.3 Extraction of chromosomal DNA from *C. acetobutylicum* P 262. A modification of the method of Marmur (1961) according to Zappe et al. (1986) was used. A dilute spore stock (1:600; 500 µl) solution was heat shocked at 75°C for 2 min, cooled to room temperature, and chilled on ice for 5 min. An aliquot (5 µl) was inoculated into CBM medium (10 ml) in Hungate tubes, and

incubated at 37°C for approximately 9 h in a water bath in order to get an A_{600} 0.3. An aliquot (30 ml) of this log-phase culture was inoculated into CBM medium (1.2 l) prepared in a 2 l Schott bottle. Incubation was continued with occasional swirling at 37°C under anaerobic conditions until A_{600} reached 0.5-0.6. Cells were harvested by centrifugation with a Beckman JA14 or a Sorvall GSA rotor at 10,000 rpm for 20 min using 250 ml bottles. Cell pellets were resuspended in CBM (40 ml) containing 10% (w/v) sucrose, 12.5 mM $CaCl_2$, 12.5 mM $MgCl_2$ and lysozyme (5 mg/ml) anaerobically. During incubation at 37°C the development of protoplasts was monitored microscopically. Approximately 90% of the protoplasts were obtained in 1-1.5 h incubation. SDS (2% w/v final concentration) and EDTA (0.05 M final concentration) were added to the remaining cultures aerobically and the lysed mixtures were extracted immediately with an equal volume of hot phenol (50°C, 0.1 M Tris-HCl pH 8.0 equilibrated). The layers were emulsified by gentle mixing for 10 min. The phases were separated by centrifugation with Beckman JA21 or Sorvall SS34 rotor at 15,000 rpm for 15 min at 4°C. The organic layers were back-extracted with 0.5 volume of TE (10 mM Tris-HCl, 1mM EDTA pH 8.0). The aqueous layers were combined together and extracted once with phenol/chlorophorm/isoamyl alcohol (25:24:1 (v/v/v). The phases were separated by centrifugation with a Beckman JA14 or Sorvall GSA rotor at 10,000 rpm for 10 min. The chromosomal DNA was precipitated by 0.1 volume of 5 M $NaClO_4$ and 0.6 volume of propan-2-ol. Chromosomal DNA was spooled using an end sealed pasteur pipette, washed with 70% ethanol and redissolved in TE buffer overnight at room temperature with gentle shaking. DNase-free RNase (100 μg/ml, final concentration) was added and incubation at 37°C continued for 30 min the DNA solution was extracted with phenol/chloroform/isoamyl alcohol again and then precipitated. The chromosomal DNA was finally

resuspended in TE buffer and its concentration was determined by the A_{260}. Aliquots of DNA solution were stored at 4°C.

A.1.4 Restriction endonuclease digestion. Restriction endonuclease digestion was carried out as described by Maniatis et al., (1982). Restriction endonuclease digestions were performed in a total volume of 20 µl in the presence of 3 units of restriction endonuclease enzyme per µg of DNA. Restriction endonuclease buffers obtained from the suppliers of the restriction endonucleases (Boehringer Manheim and Amersham), were used as recommended. Restriction endonuclease digestions were incubated at the recommended temperatures for 1-5 h. For electrophoretic analysis, the digestions were terminated by the addition of 5 µl DNA loading solution (Appendix A.2.3) to the 20 µl digestions. If the digestion products were to be ligated, they were purified by a phenol-ether extraction. The digestion products were diluted with sterile distilled water (380 µl), and TE-saturated phenol was added (40 µl; Appendix A.2.7). After vortexing briefly, the phenol was completely removed by extracting several times with water-saturated ether. The DNA was precipitated from the aqueous phase by the addition of one-tenth volume of 3 M sodium acetate (pH 4.8), and 2 volumes of 95% ethanol, cooling to -70°C for 5 min, and centrifuging for 30 min in a microfuge at 4°C. After centrifugation the pellet was washed with 70% ethanol, dried and resuspended in TE buffer.

A.1.5 Agarose gel electrophoresis. Agarose gel electrophoresis was carried out using a horizontal submerged gel system. Tris-borate EDTA (TBE) or Tris-acetate EDTA (TAE) buffers (Appendix A.2.3) were used routinely. Sigma type II agarose was used at varying concentrations (0.8%-1.2%). The amount of DNA loaded per lane also varied with the sizes and number of fragments, but under normal circumstances about 300 ng of plasmid DNA was used. The samples

were electrophoresed at 2 V per cm for 16 h and the gels were stained in electrophoresis buffer containing EtBr (0.5 μg/ml) for 15-30 min. DNA bands were visualized using a 254 nm transilluminator. A 310 nm transilluminator was used if the DNA was to be recovered from the gel.

Gels were photographed using a Polaroid CU-5 Land camera fitted with a red filter and a fixed focal length attachment. Polaroid type 667 film (ASA 3 000) was used with an exposure time of 1-2 sec at f4.7. If a negative was required then a Polaroid type 665 film (ASA 64) with an exposure of 120-140 sec at f4.7 was used.

DNA fragments were sized according to standard curves prepared by plotting the mobility against the log molecular mass of DNA fragments of known mass. Standard DNA fragments were obtained by the restriction endonuclease digestion of lambda DNA with *Pst*I, *Hin*dIII or *Eco*RI.

A.1.6 DNA ligation reactions. DNA ligation reactions were of two basic types: recircularization of plasmids for the isolation of deletion clones (use low DNA concentrations, 1 pmole DNA/ml) and recombination reactions when subcloning insert fragments into vectors (use 5-15 pmole DNA/ml). DNA concentration was calculated using the formula:

$$1 \text{ pmole} = (0.662 \times \text{kb})\text{ug}$$

Vector and insert DNA were added to the ligation reactions at a molar ratio of 1:2. Ligation reactions containing DNA, ligation buffer (Appendix A.2.7), ATP (Appendix A.2.7) and water to the required volume, were performed in sterile microfuge tubes. Sticky-end ligations were performed at room temperature for 3 h or at 15^{o}C overnight using 0.1-0.25 units of ligase, whereas blunt-end ligations were performed at room temperature for 3-20 h using 20-100 x more ligase.

A.1.8 Rapid subcloning protocol using gel purification. The rapid subcloning protocol of Struhl (1985) was used. the DNA fragments were separated by electrophoresis through low melting point (LMP) agarose (0.8%) (Seaplaque^R) in Tris-acetate buffer (50 mM, pH 8.2, no EDTA, no EtBr). The gel was stained with EtBr after electrophoresis and the DNA bands were viewed under UV light (310 nm), as briefly as possible. The desired bands were excised using a sterile scalpel blade, in as small volume as possible. The gel slices were melted at 70^{o}C for 5 min in a microfuge tube and the required amounts (2 µl vector DNA, 8 µl insert DNA) were added hot to the prepared ligation mixture containing ligation buffer, ATP, ligase and water (10 µl). The ligation was incubated at room temperature for 3 h. Before transformation of *E. coli* competent cells, the gelled ligation reactions were melted at 70^{o}C for 5 min, and then diluted with 4 volumes of TSB solution (Appendix A.2.).

A.1.9 The preparation and transformation of competent *E. coli* cells. *E. coli* cells were made competent for DNA uptake according to the method of Chung and Miller (1988). A 1/100 dilution of an overnight *E. coli* culture in LB was inoculated into 25 ml prewarmed LB and incubated at 37^{o}C, with shaking, until the culture had reached early exponential phase (A_{600}=0.3-0.6) (2-4 h). The cell culture was poured into a pre-cooled sterile SS34 tube and the cells were harvested at 1000 x g for 5 min at 4^{o}C. The cell pellet was resuspended in 2.5 ml (1/10 volume) ice-cold transformation and storage buffer (TSB) (Appendix A.2.3) and held on ice for 10 min. The *E. coli* cells (100 µl) were then mixed with DNA (routinely 50 ng) and held on ice for a further 30 min. TSB solution (0.9 ml) containing glucose (20 mM) was added to each transformation mixture and incubated at 37^{o}C for 60 min, to allow expression of the plasmid borne antibiotic marker.

A.1.10 Determination of GS activity by the g-glutamyl transferase assay. The γ-glutamyl transferase (GGT) assay, described by Bender et al., (1977) was used to measure GS activity in either *E. coli* or C. *acetobutylicum* cells. These cells were grown in the respective growth media for different times, harvested by centrifugation (5,000 rpm; Beckmann SS34) and washed with 0.8% NaCl. After centrifugation, the cells were resuspended in 0.9 ml resuspension buffer (20 mM imidazole pH 7.15, 10 mM $MgCl_2$, 2 mM β-mercaptoethanol) and treated with 0.1 ml of hexadecyltrimethylammonium bromide 10 mg/ml to inhibit further growth and render the cells permeable. Generally a dilution series was made using the previous resuspension buffer. Samples of 0.1 ml were added to 0.4 ml GGT-assay reaction buffer (Appendix A.2.4) and equilibrated at 37°C for 5 min. The reaction was initiated by the addition of 50 µl gln (0.2 M). After 15 min at 37°C, the reaction was stopped by the addition of 1 ml of stop mix (Appendix A.2.4) and the A_{600} was determined. A standard curve for this assay was plotted by using L-glutamine acid γ-monohydroxamate (GAMH; SIGMA G-2253). GS activity was expressed in moles of γ-glutamyl hydroxamate produced per min per mg of protein.

A.1.10.1 Determination of protein concentrations. Protein concentrations in solutions were determined by the method of Lowry et al. (1951). The reaction contained protein solution (0.1 ml), 0.1 M NaOH (0.1 ml) and 1 ml Folin-Lowry solution A (Appendix A.2.5). After 10 min at room temperature, 0.1 ml of Folin-Lowry solution B (Appendix B) was added followed by vigorous shaking. After 30 min at room temperature, the A_{600} was determined. Protein concentrations were calculated using a standard curve (BSA fraction V; 0.05 - 0.7 mg/ml). Protein samples were diluted such that A_{600} values were in the range 0.2 - 0.8.

A.1.11 Nucleotide sequencing

A.1.11.1 Primer annealing reaction. The supercoiled DNA (6-10 mγ, in TE buffer) was diluted to a final volume of 20 µl in distilled water. Alkaline denaturation in 0.2 N NaOH (5 min at room temperature) was followed by the addition of 5 µl of 3 M sodium acetate (pH 5.2), 25 µl of distilled water and 150 µl of chilled ethanol. This mixture was chilled to -70°C, centrifuged at 4°C for 20 min in a microfuge and washed with 200 µl of ethanol (70%). The DNA pellet was dried and resuspended in a final volume of 10 µl of sequencing buffer (40 mM Tris-HCl, pH 7.5; 20 mM $MgCl_2$; 50 mM NaCl) and 12 ng of primer. This mixture was annealed for 30 min at 40°C immediately prior to sequencing. The forward sequencing primer as supplied in the Sequenase DNA sequencing kit (US Biochemical Corp., Cleaveland, Ohio) and the M13 reverse sequencing primer (Amersham) were used, unless otherwise stated.

A.1.11.2 Sequencing reactions. DNA sequencing was done by the dideoxynucleotide triphosphate chain termination method of sanger et al., (1977) according to the protocol of Tabor and Richardson (1987), using T7 DNA polymerase and a Sequenase sequencing kit supplied by the US Biochemical Corporation, Cleaveland, Ohio. The DNA chain was radiolabelled with [α-^{35}S]dATP (1200 Ci/mmol; Amersham).

A.1.11.3 Gel electrophoresis and autoradiography. The sequencing reactions were analyzed on standard 6% denaturing acrylamide urea sequencing gels. The composition and running conditions of the gels were as described in the Amersham M13 Sequencing Handbook. After electrophoresis the gels (0.2 mm

thick) were dried onto Whatman No. 3 filter paper using a Dual Temperature Slab Gel Dryer (model 1125B; Hoefer Scientific Instruments, San Francisco). Gels containing ^{35}S-labelled DNA were placed under XAR-5 autoradiographic film and exposed for 1-2 dayss. The autoradiographs were developed using Kodak GBX X-ray developer and fixer.

A.1.12 Extraction of RNA from *E. coli* and *C. acetobutylicum* cells. The RNA extraction from these cells was performed according to the method described by Aiba et al., (1981). *C. acetobutylicum* cells were grown in GSMM and *E. coli* cells were grown in M9 or LB media, and harvested at the specified growth estimated by the A_{600}, respectively. The cells were resuspended in 3 ml of a solution of 0.02 M sodium acetate (pH 5.5), 0.5% SDS, and 1 mM EDTA. After addition of 3 ml of redistilled phenol (equilibrated in 0.02 M sodium acetate, pH 5.5), the mixture was incubated at 60°C for 5 min with gentle shaking. After centrifugation the aqueous phase was re-extracted by phenol. The RNA in the aqueous phase was ethanol precipitated (Appendix 1.12.1) and chilled at -70°C for 30 min. The RNA precipitate was collected by centrifugation and redissolved in 3 ml of the same acetate/SDS buffer. The ethanol precipitation was repeated two more times. The final precipitate was dissolved in 1 ml of distilled water. The RNA concentration was determined by measuring the A_{260}.

A.1.12.1 Ethanol precipitation of RNA. To precipitate RNA efficiently, NH_4OAc was added to a final concentration of 0.5 M before addition of 2.5 volumes of ethanol, or to 0.145 M if the RNA was already in ethanol. After adjusting the salt concentration, the RNA was precipitated by addition of 2.5 volumes of ethanol. The RNA-ethanol solutions were chilled for at least 15 min at -70°C. The RNA was recovered by centrifugation of the samples at 10,000 rpm for 15 min at 4°C. All tubes were positioned the same way during the spin,

so the pellets were formed at the back of the tubes. The ethanol supernatant was poured off, in one smooth motion, from the opposite side of the tube from where the pellet was formed. The rim of the inverted tube was blotted into absorbent paper, drained for a few min and re-blotted. Finally, the pellet was dried by placing the tube upright in a 42°C incubator for about 15 min. The RNA was stored for short-term use in 0.5 M NH_4OAc, and for longer term storage in 2.5 volumes of ethanol.

A.1.13. Preparation of template DNA. Synthesis of RNA molecules to be used as riboprobes were carried out by run off transcription from plasmid DNA templates. The template DNA was linearized by digestion with a restriction endonuclease that cleaves in a site downstream of the insert DNA to be transcribed. The DNA templates were linearized using a restriction site in the polylinker on the amino-terminal side of the coding region of the protein to synthesize mRNA, or other RNA complementary (antisense) transcripts. The template DNA was usually digested with proteinase K (100-200 µg/ml for 30 min at 50°C) and then phenol/chloroform extracted.

A.1.13.1 *In vitro* transcription reaction. The *in vitro* transcription reactions were performed under standard conditions according to the maxiscript SP6 *in vitro* transcription kit supplied by Ambion, Inc., Austin, Texas. The composition and the conditions were as described in the instruction manual. For the synthesis of radioactive probes [α-^{32}P] UTP or CTP was used at approximately 400-800 Ci/mmole. The concentration of the limiting nucleotide was 5-25 µM to synthesize transcripts of a size greater than 300 nucleotides. The components of the reaction where assembled at room temperature in a volume of 20 µl containing template DNA, DTT, ATP, CTP, GTP, transcription buffer, RNase

inhibitor [α-^{32}P] UTP, and SP6 or T3 RNA polymerase using 100 units per reaction. The reaction mixture was incubated at 25°C for 30-60 min.

A.1.13.3 Removal of DNA template. The removal of the DNA template from the transcription reaction was accomplished by treatment with RNase-free DNase I. DNase I was added to the reaction mixture and incubated for 15 min at 37°C. The reaction was stopped by adding EDTA 0.2 M and RNase free water.

A.1.13.4 Gel purification of the probe. At the end of the DNase I incubation an equal volume of gel loading buffer was added to the reaction. The tube was heated for 3-5 min at 80-90°C. An alicuot (2 µl) was removed for TCA preciptitation to determine the specific activity of the radiolabeled product. All the remaining reaction was loaded on a 0.75 mm thick 8 M urea 55 acrylamide gel and run for 1 h at 300 volts. pBR322 digested with *Msp*I endonuclease was used as a standard DNA for approximate size determination of the transcription products. After electrophoresis, the gel was covered with plastic wrap and exposed to X-ray film for about 5-10 min. After exposure, the film was developed and used to precisely localize the area of the gel that contained the full length labeled transcript. The area of the gel containing the transcript was excised and transferred to a microfuge tube and submerged in about 350 µl of elution buffer and incubated at 37°C for 12 h. About 95% of the label diffused out of the gel into the surrounding elution buffer. The amount of radioactive label in an aliquot of the eluted probe was determined by scintillation counting. The probe was stored in elution buffer at -20°C.

A.1.13.5 Calculation of probe specific activity. Considering a 20 µl transcription reaction contained 5 µl of ^{32}P-UTP (800 Ci/mN, 10 mCi/ml) and

2μl of 50 μM unlabeled UTP. A 2 μl aliquot of the final reaction was removed and diluted into 198 μl of TE containing 100 μg of carrier RNA; 100 μl of this dilution is counted directly in a scintillation counter and found to contain 2.6 x 10^6 cpm. The proportion of of ^{32}P incorporated into RNA is determined by TCA precipitation and filtration of the remaining 100 μl of the diluted reaction. The counting efficiency of the ^{32}P isotope in liquid scintillation cocktail is assumed to be 100%. The specific activity of the RNA probe is calculated as follows:

1. Proportion of UTP incorporated into RNA. Assuming the proportion of [α-^{32}P]UTP incorporated reflects the proportion of labeled and unlabeled UTP incorporated:

$$x\ 10^6 \text{ cpm TCA} / 3 \times 10^6 \text{ cpm} = 50\%$$

2. Moles of [α-^{32}P]UTP in the reaction. Calculated by the conversion of the volume of [α-^{32}P]UTP added (6 μl) to the number of mCi of ^{32}P added, and then conversion of the amount of [α-^{32}P]UTP in mCi to a molar amount using the known specific activity and concentration of the [α-^{32}P]UTP (800 Ci/mmole, 10 mCi/ml).

mCi [α-^{32}P]UTP in reaction

$$= (6\ \mu l \times 10\ mCi)/ml \times 1\ ml/1000\ \mu l$$
$$= 0.06\ mCi$$

mmoles of [α-^{32}P]UTP

$$= (0.06\ mCi \times 1\ mmole) / 800\ Ci \times 1\ Ci / 1000\ mCi$$
$$= 0.06 / (8 \times 10^5\ mmoles)$$
$$= 0.0075 \times 10^{-5}\ mmoles$$
$$= 0.075\ nmoles\ in\ reaction$$

3. Moles of unlabeled UTP in the reaction

$$50\ \mu moles/ 1000\ ml \times 2\ \mu l \times 1\ ml/ 1000\ \mu l = 0.1 \times 10^{-3} \mu moles$$
$$= 0.1\ nmoles$$

4. Total UTP in the reaction.

0.0075nmoles [α-^{32}P]UTP + 0.1nmoles cold UTP = 0.175 nmoles total UTP

5. Total UTP incorporated into RNA.

nmoles in reaction x 50% incorporation = 0.0875 nmoles incorporated

6. RNA synthesized in nanograms. RNA synthesized was assumed to contain equal molar amounts of all four ribonucleotides. Therefore one can assume 0.0875 nmoles of each ribonucleotide was incorporated. The sum of the molecular weights of the four ribonucleotides is about 1320 daltons. (the average molecular weight of a nucleotide in RNA is 330 daltons.)

ng synthesized = 1320×10^9 ng/ 10^9nmoles x 0.0875 nmole = 115.5 ng

7. cpm incorporated into the RNA product. the final reaction volume from which the 2 µl sample was removed to determine label incorporation was 42 µl (20 µl transcription reaction + 1 µl DNase + 21 µl loading buffer). The amount of sample TCA precipitated and found to contain 1.5×10^6 cpm was 1 µl (half of the 2 µl sample diluted 1:100 in 198 µl TE and carrier). So the whole reaction contained 42 µl x 1.5×10^6 cpm/µl = 63×10^6 cpm/µl = 63×10^6cpm of TCA-precipitable material.

8. Specific activity of the product = 63×10^6 cpm/ 115.5 ng
= 5.45×10^5 cpm/ ng
= 5.45×10^8 cpm/ µg

A.1.14 RNase protection assay. The RNase protection assays were performed according to the procedures described by the Ambion Ribonuclease Protection

Assay kit (Ambion, Inc. Austin, Texas). The composition and assay conditions were as described in the instruction manual.

A.1.14.1. Hybridization of the ribonucleoproe and the sample RNA. Usually an amount of probe was assumed to be in molar excess over the target mRNA was utilized. About 150-900 pg or 2 x 10^4 - 1.2 x 10^5 cpm of gel purified-^{32}P labelled probe were mixed with different amounts of the sample RNA (usually about 1 - 10 µg) in a 1.5 ml microfuge tube. Controls of yeast RNA mixed with the same amount of probe were included, with or without RNase. The samples were ethanol precipitated (Appendix A.1.11.1) and the tubes were placed at -70°C for 15 min and microfuged at 10,000 rpm for 15 min at 4°C. The pellets were resuspended in 20 µl of hybridization buffer by thoroughly vortexing and briefly centrifuging tubes. All the tubes were heated at 90°C for 3-4 min and then submerged in a 45°C heat block overnight.

A.1.14.2 RNase digestion of hybridized RNAs. The tubes were removed from the 45°C heat block and microfuged briefly to precipitate the condensation present on the sides. A mixture of concentrated RNase A and RNase T1 were diluted in RNase digestion buffer in a 1:50 ratio. Diluted RNase A/T1 solution was added to all the experimental tubes and to one of the yeast RNA control tubes. In the other yeast RNA control tube this solution was replaced with RNase digestion buffer. All tubes were vortexed and microfuged briefly and incubated at 37°C for 30 min. A SDS solution 20% (w/v) was mixed with an equal volume of a Protein kinase K/yeast RNA/glycerol 50% (v/v) solution. Twenty µl of this mixture were added to all tubes. The tubes were vortexed, microfuged briefly and incubated for 15 min at 37°C. A volume of 250 ul of phenol/$CHCl_3$/isoamyl alcohol (25:24:1) was added to each tube, which were vortexed thoroughly and microfuged for at least one min to completely separate

phases. The aqueous phases were removed to new tubes and 625 µl of ethanol 100% (v/v) were added to each tube. The tubes were vortexed and stored at -70°C for 15 min.

A.1.14.3 Separation and detection of protected fragments. A suitable denaturing polyacrylamide gel for the separation of the protected fragments was prepared. The tubes (stored at -70°C) were removed from the freezer and microfuged for 15 min at 4°C. The ethanol supernatant was carefully removed from each tube. Each pellet was resuspended in 8 µl of loading buffer by vigorous vortexing and brief microfuging. All the tubes were heated for 4 min at 90°C, re-vortexed and re-microfuged briefly. The samples were loaded on a 5% acrylamyde 8 M urea gel. The composition and running conditions of the gels were as described in the Ribonuclease protection assay Ambion kit (catalog No. 1400). pBR322 digested with *Msp*I endonuclease was included as standard DNA for size determinations. The electrophoresis was run at 250 volts for about 1 h in Tris-borate buffer. Finally, the gel was transfered to chromatography paper Whatman No.3 ,covered with plastic wrap, and exposed to Xar-5 autoradiographic film ray film at -70°C. The autoradiographs were developed using Kodak GBX X-ray developer and fixer.

A.1.15. Molecular titraton assay. The previously described *in vitro* synthesized-[^{32}P] labelled ribonucleoprobe was used in tracer excess titrations to quantify the number of target RNA transcripts. The method utilized was previously described by Lee and Costlow, (1987). The titration assay consisted of a series of hybridizations between a constant amount of RNA probe and increasing amounts of input cellular RNA. Five data points were usually required to produce a line from which the slope can be accurately determined. Ribonuclease A and T1 cleave 3' to pyrimidines and G residues, respectively.

The specificity of these ribonucleases for single stranded RNA and their high activity in simple buffers make them very useful for the separation of hybridized probe (double-stranded RNA) from unreacted probe (single-stranded RNA) in a titration assay. Once hybridizations and RNase digestions of the protected fragments were performed as described in the previous sections (Appendix 1.13.1-2), these procedures were followed by precipitation in 0.5 M sodium phosphate buffer, pH 7.0. The data were expressed as RNase resistant counts per min versus total RNA input. The background hybridization was substracted from each of the raw data points of the assay, which was represented by the y intercept of the plot. The data points were then replotted to determine the slope. The slope has the units cpm of ribonuclease-resistant probe per ug of total cellular RNA. Using the specific activity of the probe and the length of the hybridazable portion of each riboprobe, a relationship between the number of transcripts and the slopes of the plots can be established according to the following formula:

$$T = k' m / \beta \delta, \quad k' = n \Sigma / \alpha$$

where T is the number of transcripts per cell, m is the experimentally determined slope of the titration curve, β is the probe specific activity (dpm/ng) and δ is the molecular weight of the hybridizable portion of the probe (ng/mmol), and k' is a constant involving: the Avogadro's constant n, the mass of RNA per cell Σ, and the ^{32}P fractional counting efficiency α.

A.1.16. DNA alkali blotting procedure. DNA fragments resolved by gel electrophoresis were transfered to a Hybond N^+ hybridization membrane (Amersham) essentially by the protocol of Reed and Mann (1985). The use of a nylon transfer membrane allows the capillary transfer of DNA restriction fragments in alkali rather than in neutral, high ionic strength solvents (used in

conventional Southern transfer), and eliminates the need for post-transfer fixation (Reed and Mann, 1985). After electrophoresis the gel was rinsed in 2 volumes of HCl (0.25 M) for 20 min at room temperature with gentle agitation, followed by a brief rinse in distilled water. The gel was then placed on top of 2 sheets of Whatman 3 MM filter paper (wetted with 0.4 N NaOH, and placed on top of an inverted gel-casting tray in a plastic box, such that the filter paper touched the base of the box, forming a wick), and was flooded with 50-100 ml of 0.4 N NaOH. A sheet of Hybond N^+ (wetted by floating onto and then immersion in distilled water) was placed on top of the gel, and any air bubbles were removed. Three sheets of Whatman 3MM fiter paper, wetted in 0.4 N NaOH, were laid onto the membrane, followed by a 4 cm thick layer of absorbent paper. A light weight was placed on top of this, and transfer left to continue overnight. After transfer, the membrane was rinsed briefly with gentle agitation in 2 x SSC (Appendix A.2.6). The membrane was now ready for hybridization or could be wrapped in saran wrap and stored at 4^oC.

A.1.17. Hybridization and washing procedures. The Hybond N^+ membrane to which the DNA fragments were transfered (previous section A.1.15), was sealed in a plastic bag and prehybridized with the prewarmed hybridization solution 6 x SSC, 0.5% SDS, 5 x Denhardt's solution, and 100 µg/ml denatured-fragmented salmon sperm DNA, at 68^oC for 30 min with good agitation. The nick-translated radioactive DNA probe was denatured by boiling for 5 min before adding into the hybridization bag. A total count of 10^6 cpm/ml radioactive DNA probe was usually used. Hybridization was carried out at 68^oC overnight with good agitation. After hybridization, the membranes were washed twice with 2x SSC, 0.5% SDS at room temperature for 15 min. A final wash with 0.1% x SSC, 0.1% SDS was performed at different temperatures for the different stringent conditions required. The highest stringent wash was

obtained by washing at 68°C for 15 min. The membrane was then air-dried briefly, sealed in a plastic bag and autoradiographed at -70°C for various exposure times.

A.2 MEDIA, BUFFERS AND SOLUTIONS

All media, buffers, and solutions were sterilized by autoclaving at 121°C for 20 min. Heat labile substances were steriilized by filtration through 0.22 µm membrane filters (Millipore).

A.2.1 Media

Glucose-Mineral Salts-Biotin medium (GSMM) (Holdeman et al., 1977).

Ingredient	Amount/l
Glucose	10.0 g
$(NH_4)_2SO_4$	1.0 g
Salts stock solution[a]	4.0 ml
Biotin (0.0002% w/v)	250.0 µl
$NaHCO_3$	1.0 g
Cysteine HCl	0.5 g
Resazurin stock solution (0.02% w/v)	10.0 ml

[a]Salts stock solutions contained/l

$CaCl_2$	0.2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
K_2HPO_4	1.0 g
KH_2PO_4	1.0 g
$NaHCO_3$	10.0 g
NaCl	2.0 g

***Clostridium* basal medium (CBM)** (O'Brien and Morris, 1971)

Ingredient	Amount/l
Glucose	10.0 g
Casein hydrolysate	4.0 g
Yeast extract	4.0 g
$MgSO_4 \cdot 7H_2O$ (20% w/v)	1.0 ml
$MnSO_4 \cdot 4H_2O$ (1% w/v)	1.0 ml
$FeSO_4 \cdot 7H_2O$ (1% w/v)	1.0 ml
p-Aminobenzoic acid (0.1% w/v)	1.0 ml
Thiamine HCl (0.1% w/v)	1.0 ml
Biotin (0.0002% w/v)	250.0 µl
$NaHCO_3$	1.0 g
Cysteine HCl	0.5 g
Resazurin stock solution (0.02% w/v)	10.0 ml

Cysteine HCl (10.0 ml) and $NaHCO_3$ (20 ml) were added as sterile stock solutions after the medium had been autoclaved and prior to pouring.

Minimal M9 medium (*E. coli*) (Miller et al., 1972)

Ingredient	Amount/l
Glucose (20% w/v)	10.0 ml
M9 salts[a]	100.0 ml
$MgSO_4$ (1 M)	1.0 ml
$CaCl_2$ (0.1 M)	1.0 ml
Thiamine-HCl (1 M)	1.0 ml

[a]M9 salts contained/l:

Na_2PO_4	60.0 g
KH_2PO_4	30.0 g
NH_4Cl	10.0 g
NaCl	5.0 g

Luria-Bertani medium (LB):

Ingredient	Amount/l
Bactotryptone	10 g
Yeast extract	5 g
NaCl	5 g
Distilled water	1000 ml

Solid media contained 1.5% (w/v) agar.

A.2.2 Media additives

Media were cooled to 50°C before addition of antibiotics, XGal, or IPTG. Plates containing these additives were made fresh before use.

Antibiotics

Antibiotic stock solutions:

Ampicillin	100 mg/ml in water
Chloramphenicol	20 mg/ml in ethanol (96%)
Kanamycin	62.5 mg/ml in water
Tetracycline	12.5 mg/ml in ethanol (50%)

All antibiotics were filter sterilized and stored at -20°C.

IPTG (isopropyl-β-D-thio-galactopyranoside)

IPTG (100 mM)	23.4 mg
Distilled water	1.0 ml

This solution was stored in aliquots at -70°C.

XGal (5-bromo-4-chloro-3-indolyl-β-galactoside)

Xgal (0.2% w/v)	0.2 g
Dimethylformamide	10.0 ml

This solution was stored at -70°C.

A.2.3 Buffers and solutions

DNA loading solution (6x)

Bromophenol blue	250.0 mg
Sucrose	40.0 g
Distilled water	to 100.0 ml

This solution was stored at 4°C.

Tris acetate buffer (50x)

Tris-base	242.0 g
Acetic acid	57.1 ml
EDTA (0.5 M, pH 8.0)	100.0 ml
Distilled water	to 1000.0 ml

Tris borate buffer (5x)

Tris-base	54.0 g
Boric acid	27.5 g
EDTA (0.5 M, pH 8.0)	20.0 ml
Distilled water	to 1000.0 ml

TSB solution

LB	150.0 ml
pH to 6.1 with 2 drops conc. HCl	
PEG 4000	15.0 g
$MgSO_4$ (1 M)	1.5 ml
$MgCl_2$ (1 M)	1.5 ml

Dispensed in 20 ml aliquots and autoclaved. DMSO (1 ml) and glucose (0.5 M, 400 µl when required) were added immediately before use.

A.2.4. Buffers for the g-glutamyl transferase (GGT) assay

Stocks solutions

Imidazol buffer (1 M)	6.808 g/100 ml
Hydroxylamine - HCl (0.8 M)	5.560 g/100 ml
$MnCl_2$ (0.1 M)	1.979 g/100 ml
Potassium arsenate (0.28 M)	5.040 g/100 ml
Glutamine (0.2 M)	0.292 g/10 ml
ADP (0.1 M) (sodium-salt)	0.017 g/ml

Resuspension buffer

Stock solution	per 200 ml	Final conc.
Imidazol 1 M, pH 7.15	4 ml	20.0 mM
$MgCl_2 \cdot 6H_2O$	2 ml	10.0 mM
β-mercaptoethanol (14 M)	28 µl	2.0 mM

The buffer was stored at 4°C.

Assay reaction buffer

Stock solution	per 20 ml	Final conc.
Imidazole (1M)	3.71 ml	185.00 mM
Hydroxylamine (0.8 M)	0.61 ml	24.50 mM
$MnCl_2$ (0.1 M)	74 µl	0.37 mM
Potassium arsenate (0.28 M)	2.47 ml	35.00 mM
ADP (0.28 M) (sodium salt)	247 µl	3.50 mM
Distilled water	12.90 ml	-

Reaction termination solution

$FeCl_3 \cdot 6H_2O$	55 g
Trichloroacetic acid (TCA)	20 g
HCl (concentrated)	21 ml
Distilled water	to 1000 ml

A.2.5 Folin-Lowry assay solutions

Na_2CO_3 (2%, w/v)	1 g
Sodium citrate (2%, w/v)	0.5 ml
$CuSO_4 \cdot 5H_2O$ (1%, w/v)	0.5 ml
Distilled water	to 50.0 ml
Folin & Ciocalteu's phenol reagent	5.0 ml
Distilled water	5.0 ml

A.2.6 DNA hybridization solutions

Alkali fixation solution

NaOH (0.4 M)	16.0 g
Distilled water	to 1000.0 ml

Denaturing solution

NaCl (1.5 M)	87.6 g
NaOH (0.5 M)	20.0 g
Distilled water	to 1000.0 ml

Denhardt's solution (50x)

Bovine serum albumin (1% w/v)	1.0 g
Ficoll (1% w/v)	1.0 g
Polyvinylpyrrolidone-40	1.0 g
Distilled water	to 1000.0 ml

This solution was stored in aliquots (10 ml each) at -20°C.

Hydrolysing solution

HCl, concentrated (0.25 M)	26.0 ml
Distilled water	to 1000.0 ml

Neutralizing solution

EDTA (1.0 mM)	372.0 mg
NaCl (1.5 M)	87.6 g
Tris-base (0.5 M)	60.5 g
Distilled water	to 1000.0 ml

This solution was adjusted to pH 7.2 with HCl.

Salmon sperm DNA

A 10 mg/ml solution was made in TE buffer. The DNA solution was sonicated at full power (20 microns) for 10 min in a MSE Soniprep sonicator. The solution was aliquoted and stored at -20$_o$C. Immediately before use the DNA was denatured by boiling for 10 min followed by cooling on ice.

SSC (20x)

NaCl (3.0 M)	175.3 g
Sodium citrate (0.3 M)	88.2 g
Distilled water	to 1000.0 ml

The pH of this solution was adjusted to 7.0 with NaOH. the solution was autoclaved.

A.2.7 General DNA manipulation solutions

ATP (10x)

Adenosine 5'-triphosphate	30.0 mg
Distilled water (sterile)	5.0 ml

The pH of this solution was adjusted to 7.0. The solution was stored in 100 µl aliquots at -70°C.

DTT (1M)

Dithiothreitol	3.1 g
Sodium acetate (10 mM, pH 5.2 sloution)	20.0 ml

This solution was filtered and stored in aliquots at -20°C.

EDTA (0.5 M, pH 8.0)

EDTA	168.1 g
Distilled water	to 1000.0 ml

The EDTA was dissolved at a pH higher than 8.0 by adding approximately 20 g of solid NaOH. The solution was autoclaved and stored at room temperature.

Ethidium bromide solution

(2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide). A solution of this salt was made in distilled water at a concentration of 10 mg/ml. The solution was stored in a dark bottle at room temperature.

Isopropanol (salt saturated)

Isopropanol was saturated with a buffer solution which consisted of 5 M NaCl, 10 mM Tris-HCl and 1 mM EDTA (pH 8.0).

Phenol (TE saturated)

Phenol (200 g) was melted at 65°C and 0.3 g 0f 8-hydroxyquinoline was added. The phenol was extracted three times with TE (10x) or until the aqueous phase was approximately pH 7.6. The phenol was stored under TE (1x) at -20°C.

Tris-EDTA (TE) buffer (100x)

Component	Amount
Tris-base	121.1 g
EDTA (0.5 M, pH 8.0 solution)	200.0 ml
Distilled water	to 1000.0 ml

The pH of this solution was adjusted to the required pH value with HCl. The solution was autoclaved, stored at 4°C, and diluted with steril water before use.

Klenow (DNA polymerase I) buffer

Stock solution	Final conc.	Amount/10ml
Tris-HCl (1 M, pH 7.6)	0.1 M	1.0 ml
$MgCl_2$ (1M)	0.1 M	1.0 ml
NaCl (5M)	0.5 M	1.0 ml
2-mercaptoethanol	0.7 M	50.0 µl
Distilled water		7.0 ml

This buffer was stored at -20°C.

Ligase dilution buffer

Stock solution	Final conc.	Amount/10ml
Tris-HCl (1 M, pH 7.6)	20 mM	0.2 ml
EDTA (0.5 M, pH 8.0)	1 mM	2.0 µl
DTT (0.5 M)	5 mM	10.0 µl
KCl (1 M)	60 mM	0.6 ml
Glycerol	44% (v/v)	4.4 ml
Distilled water		4.8 ml

This buffer was stored at -20°C.

Ligation buffer (10x)

Stock solution	Final conc.	Amount/10ml
Tris-HCl (1M, pH 7.6)	66 mM	660.0 µl
$MgCl_2$ (1M)	6 mM	66.0 µl
ATP (0.1 M)	1 mM	100.0 µl
DTT	100 mM	15.4 mg
Distilled water		174.0 µl

This buffer was stored at -70°C.

A.2.8 Restriction enzyme buffers

Stock solution	Final conc.
Tris-HCl buffer (1M, pH 7.9)	0.1 M
$MgCl_2$ (1M)	0.1 M
DTT (0.5 M)	10.0 mM
BSA (10 mg/ml)	1 mg/ml
Glycerol	44% (v/v)
NaCl (5 M)	0, 50, 100 or 150 mM

The restriction enzyme buffers were made according to the following table. These buffers were stored at -20°C.

Stock solution	Salt concentration (mM)			
	0	50	100	150
Tris-HCl (1M, pH 7.9)	1.0 ml	1.0 ml	1.0 ml	1.0 ml
$MgCl_2$ (1 M)	1.0 ml	1.0 ml	1.0 ml	1.0 ml
DTT (0.5 M)	0.2 ml	0.2 ml	0.2 ml	0.2 ml
BSA (10 m/ml)	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Glycerol	4.4 ml	4.4 ml	4.4 ml	4.4 ml
NaCl (5 M)	-	1.0 ml	2.0 ml	87.7 mg
Distilled water	2.4 ml	1.4 ml	0.4 ml	2.4 ml

Restriction enzyme dilution buffer

Stock solution	Final conc.	Amount/10 ml
Tris-HCl (1 M, pH 7.5)	10 mM	0.1 ml
KCl (1 M)	50 mM	0.1 ml
Distilled water		5.3 ml

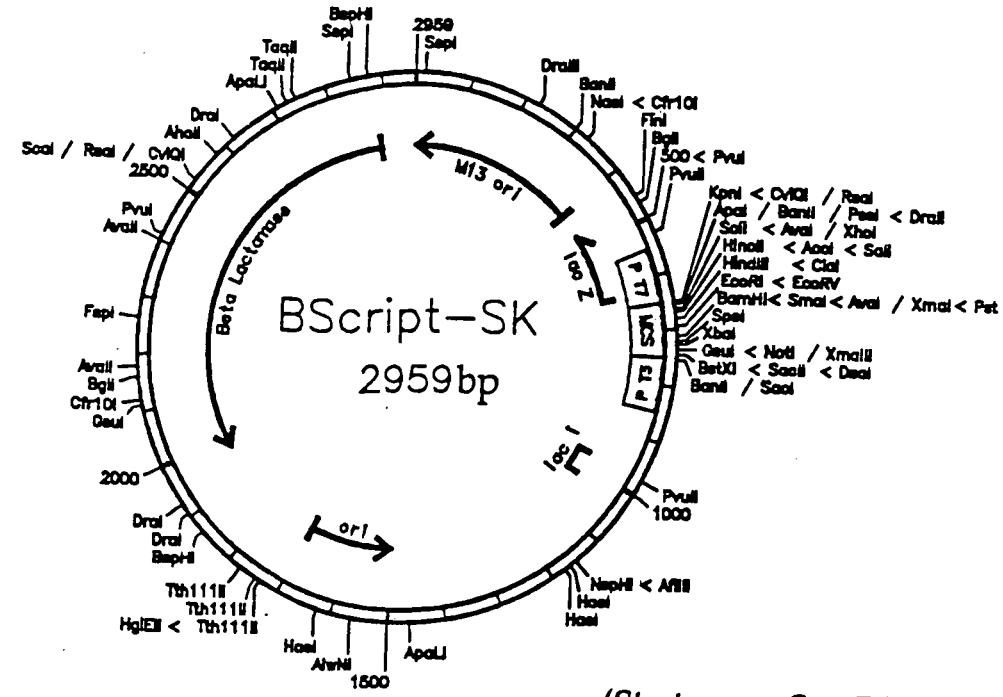
This solution was filter sterilized and the following reagents were added:

Stock solution	Final conc.	Amount/10 ml
2-mercaptoethanol	10 mM	7.0 µl
Gelatin (10 mg/ml)	100 ug/ml	0.1 ml
Glycerol	44% (v/v)	4.4 ml

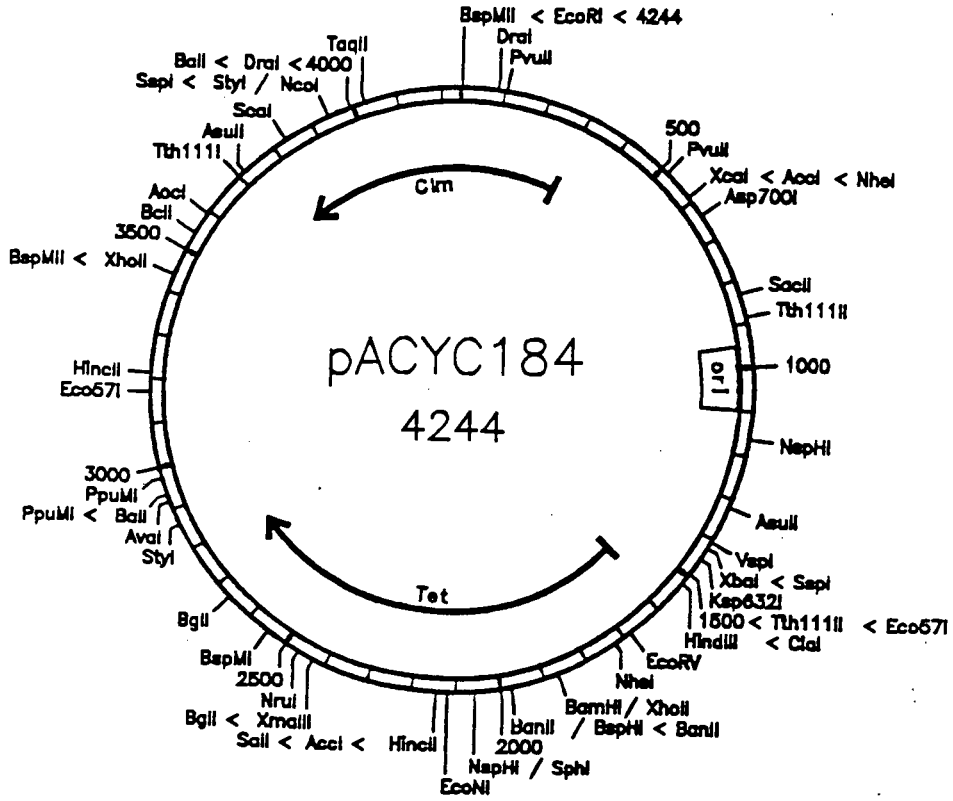
APPENDIX B

CLONING VECTORS, RESTRICTION MAPS, BACTERIAL STRAINS AND PLASMIDS

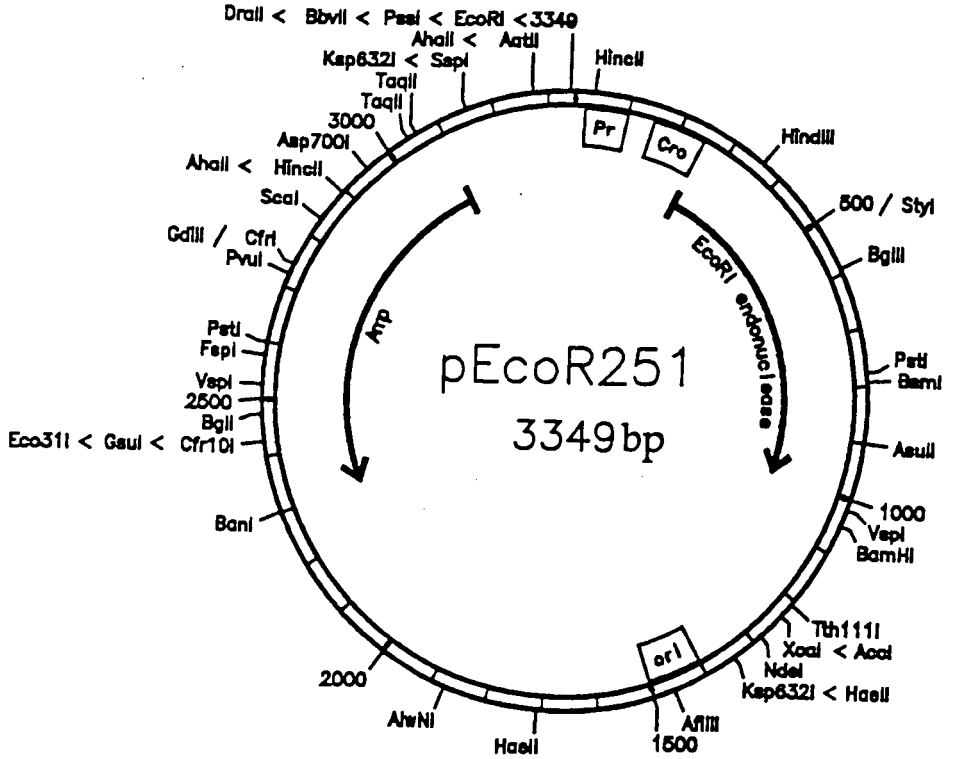
B.1. CLONING VECTORS AND RESTRICTION MAPS



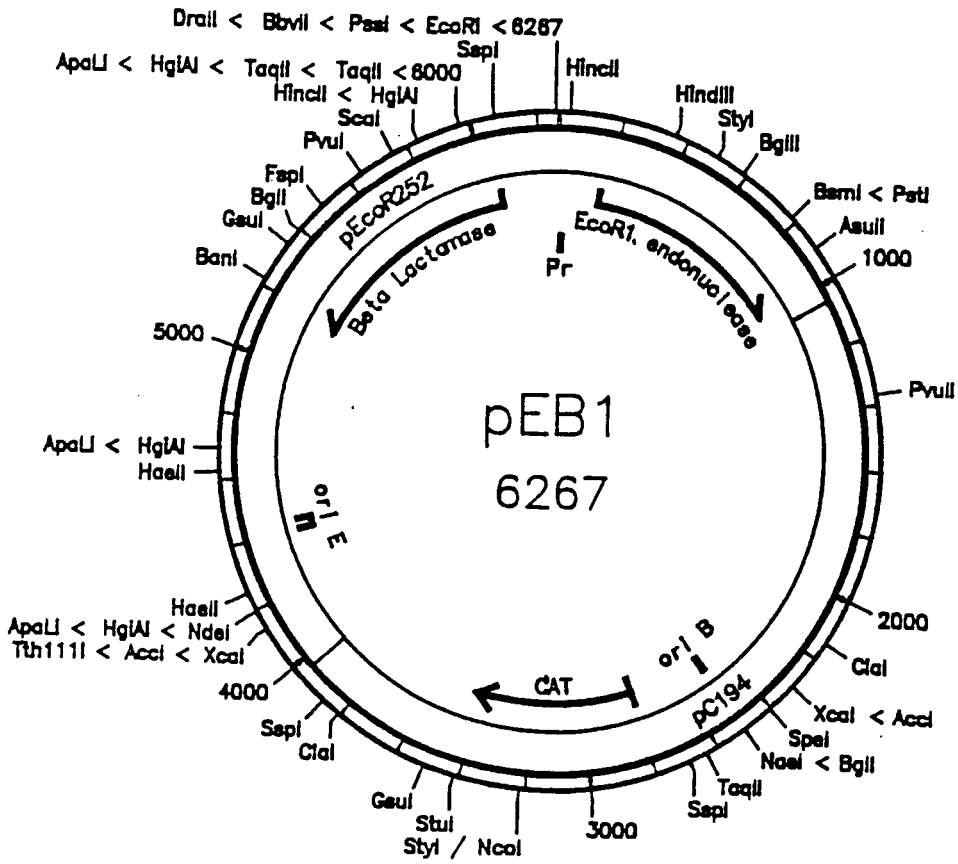
(Stratagene, San Diego)



Chang and Cohen (1978)



(Zabeau and Stanley, 1982)



(Lin *et al.*, 1990).

B.2. BACTERIAL STRAINS AND PLASMIDS

Strain or plasmid	Relevant characteristics	Reference
Strain		
E. coli YMC11	Δ*lacU*169, Δ(*glnG-glnA*)	Chen et al., 1982)
E. coli YMC10	Wild type	Backman et al., (1981)
E. coli JM109	Δ(*lac-proAB*) *lacI*^{q} Δ(*lacZ*)*M15 recA1*	Yanish-Perron et al., (1985)
C. acetobutylicum P262	Wild Type	Jones et al., (1982)
Plasmid		
pEcoR251	Ap^r	Usdin et al., (1988)
pLK34	Ap^r	Botterman and Zabeau, (1987)
pUC13	Ap^r, *lacZ*'	Messing and Vieira, (1982)
pACYC177	Kn^r, Ap^r	Chang and Cohen, (1978)
pSP64	Ap^r, SP6 polymerase promoter	Brown et al., (1986)
pBluescript-SK	Ap^r, T_3 and T_7 polymerase promoters	Stratagene, San Diego, CA. USA
pEB1	Ap^r, ori E, oriB	Lin et al., (1990)
pHZ200	*C. acetobutylicum glnA* region	Usdin et al., (1978)
pHZ202	Deletion derivative of pHZ200	Usdin et al., (1988)
pGln1300	Ap^r, *glnA*$^+$	Janssen et al., (1988)
pDR7	Ap^r, *glnA*$^+$, Δ(P_3)	Janssen (1990)
pTS-1	Kn^r, *glnA*$^+$	Janssen (1990)
pJP4	Ap^r, antisense DNA sequence, P_3	Janssen (1990)
pSPAS	Ap^r, antisense DNA sequence, P_3	This study
pSPgln700	Ap^r, internal *glnA* fragment	This study
pupglnA1300	Cm^r, *glnA*::*lacZ*	This study
pglt78	Ap^r, 5'region putative *gltS*	H. Stutz (PhD Thesis)

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