

Co-regulation of the nitrogen-assimilatory gene cluster in *Clostridium saccharobutylicum*

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Nitrogen assimilation is important during solvent production by *Clostridium saccharobutylicum* NCP262, as acetone and butanol yields are significantly affected by the nitrogen source supplied. Growth of this bacterium was dependent on the concentration of organic nitrogen supplied and the expression of the assimilatory enzymes, glutamine synthetase (GS) and glutamate synthase (GOGAT), was shown to be induced in nitrogen-limiting conditions. The regions flanking the gene encoding GS, *glnA*, were isolated from *C. saccharobutylicum* genomic DNA, and DNA sequencing revealed that the structural genes encoding the GS (*glnA*) and GOGAT (*gltA* and *gltB*) enzymes were clustered together with the *nitR* gene in the order *glnA-nitR-gltAB*. RNA analysis showed that the *glnA-nitR* and the *gltAB* genes were co-transcribed on 2.3 and 6.2 kb RNA transcripts respectively, and that all four genes were induced under the same nitrogen-limiting conditions. Complementation of an *Escherichia coli* *gltD* mutant, lacking a GOGAT small subunit, was achieved only when both the *C. saccharobutylicum* *gltA* and *gltB* genes were expressed together under anaerobic conditions. This is believed to be the first functional analysis of a gene cluster encoding the key enzymes of nitrogen assimilation, GS and GOGAT. A similar gene arrangement is seen in *Clostridium beijerinckii* NCIMB 8052, and based on the common regulatory features of the promoter regions upstream of the *glnA* operons in both species, we suggest a model for their co-ordinated regulation by an antitermination mechanism as well as antisense RNA.

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INTRODUCTION

There is renewed interest in the production of solvents and biofuels from renewable resources (Dürre, 1998; Schubert, 2006), but the economic viability of any such process is dependent on product yield. The closely related saccharolytic *Clostridium* species, *Clostridium saccharobutylicum* NCP262 and *Clostridium beijerinckii* NCIMB 8052 (both formerly known as *Clostridium acetobutylicum*; Keis *et al.*, 1995), are obligately anaerobic endospore-forming bacteria that produce acetone and butanol as fermentation end-products (Jones & Woods, 1986). *C. saccharobutylicum* NCP262 has long been recognized as one of the best industrial strains for the production of solvents, but many aspects of the fundamental metabolism of the solvent-producing clostridia remain poorly characterized. Several studies have demonstrated that solvent yields are significantly affected by the

nitrogen source (Long *et al.*, 1984; Monot & Engasser, 1983), while *C. saccharobutylicum* NCP262 did not produce solvents in ammonia-limited cultures (Gottschal & Morris, 1981; Long *et al.*, 1984).

Most bacteria possess two primary pathways for the assimilation of ammonia, the energy-dependent glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway and the energy-conserving glutamate dehydrogenase (GDH) pathway (Merrick & Edwards, 1995). The tightly coupled GS/GOGAT pathway catalyses the synthesis of glutamine and glutamate, effectively cycling these key metabolites from which nearly all other cellular nitrogen-containing compounds are derived: GS catalyses the amidation of endogenous glutamate to form glutamine, and GOGAT catalyses the transamidation of the amide group from glutamine to 2-oxoglutarate to form glutamate. GDH produces glutamate from 2-oxoglutarate. In some organisms, including *Bacillus subtilis* (Fisher & Sonenshein, 1991), *Clostridium pasteurianum* (Dainty, 1972) and *Clostridium thermoautotrophicum* (Bogdahn & Kleiner, 1986), the GS/GOGAT pathway is solely responsible for the assimilation of ammonia into organic

Abbreviations: AS, antisense; GDH, glutamate dehydrogenase; GS, glutamine synthetase; GOGAT, glutamate synthase; RR, response regulator.

The GenBank/EMBL/DDBJ accession number for the *gltAB* sequence of *Clostridium saccharobutylicum* is AF082880.

compounds. Bacteria growing with preferred nitrogen sources, i.e. those that support the fastest cell growth rate, generally contain low levels of GS and GOGAT, whereas high levels of these enzymes are present during growth under nitrogen-limited conditions to ensure adequate supplies of glutamine and glutamate.

The global regulation of these nitrogen-assimilatory enzyme activities is generally tightly controlled in response to nitrogen and energy conditions. The arrangement of nitrogen metabolism genes varies in different species and there are no reports to date in which the genes encoding GS and GOGAT, *glnA* and *gltAB* respectively, have been found to be physically linked on the genome. In the *Enterobacteriaceae*, the *glnA* gene forms an operon with the *ntrB* and *ntrC* genes which control the regulation of GS activity (Merrick & Edwards, 1995). In *B. subtilis*, three independent regulatory proteins, GlnR, TnrA and CodY, function under different nutritional conditions to control nitrogen assimilation (Fisher, 1999; Belitsky, 2002). In *Streptomyces coelicolor* and *Corynebacterium*, the *glnA* transcription unit is monocistronic, and is not adjacent to regulatory genes on the genome (Fisher, 1992; Schulz *et al.*, 2001), although AmtR has been identified as a global regulator in *Corynebacterium glutamicum* (Jakoby *et al.*, 2000).

The *C. saccharobutylicum glnA* gene, encoding GS, was isolated by functional complementation of the GS-deficient *Escherichia coli* strain YMC11 (Usdin *et al.*, 1986), and a second gene, *nitR*, encoding a putative response regulator protein, was subsequently identified downstream (Woods & Reid, 1995). The *nitR* gene encodes an antiterminator protein, which would allow RNA polymerase to read through the terminator-like structures in the promoter region of the *glnA* gene (Woods & Reid, 1995). Antisense RNA (AS-RNA), complementary to the 5' end of the *glnA* mRNA, has been implicated in the post-transcriptional inhibition of *glnA* translation under nitrogen-rich conditions (Fierro-Monti *et al.*, 1992). A gene encoding a putative GOGAT enzyme has been isolated from *C. saccharobutylicum* and was annotated *gltX*, because the deduced protein showed significant sequence identity (44%) to the *E. coli* GOGAT β -subunit (Stutz & Reid, 2004). However, RNA studies showed that *gltX* expression was not regulated by nitrogen, and it is therefore unlikely to encode a functional GOGAT enzyme.

In order to facilitate metabolic engineering of the ammonium assimilation pathways for improved solvent yields, we undertook a study of the nitrogen growth requirements of *C. saccharobutylicum* NCP262 and of the regulation of nitrogen assimilation. In this study, we characterize the chromosomal regions flanking the *glnA* gene and show that the structural genes encoding the GS and GOGAT enzymes are clustered on the genome. This is believed to be the first report of a functional gene cluster encoding both key enzymes of nitrogen assimilation. The genes are expressed under the same nitrogen-limiting

conditions, and we suggest a model for their co-ordinated regulation by an antitermination mechanism as well as by AS-RNA.

METHODS

Bacterial strains and growth conditions. Cultures of *C. saccharobutylicum* NCP262 (University of Cape Town Culture Collection) were grown at 37 °C in an anaerobic glove cabinet (Forma Scientific) in either Clostridium basal medium (CBM; Allcock *et al.*, 1982), or glucose-mineral salts-biotin minimal medium (GSMM; Holdeman *et al.*, 1977) containing various concentrations of glucose, Casamino acids (Difco), glutamine or monosodium glutamate (MSG), and inorganic nitrogen (ammonium acetate). Cell-line cultures represented cells grown to OD₆₀₀ 0.3 in CBM, washed and used at a 5% (v/v) inoculum, and spore-line cultures (10 ml) were grown directly from 5 µl spore stock heat-shocked at 70 °C for 5 min. *E. coli* strains JM105 and JM109 were grown in Luria-Bertani (LB) medium (Sambrook & Russell, 2001). *E. coli* MX3004 (*thi-1 gdh-1 pro⁺ hutC gltD227::MudIIPR13*) (Castano *et al.*, 1992) was grown on NN minimal medium (Covarrubias *et al.*, 1980).

General molecular techniques. *C. saccharobutylicum* genomic DNA was prepared according to Zappe *et al.* (1986). DNA manipulations were carried out as described by Sambrook & Russell (2001). Southern hybridization was performed using the non-radioactive DIG DNA labelling and detection protocol (Roche). DNA sequencing was performed using an ALFexpress automated DNA sequencer (Pharmacia). Sequence data were analysed using DNAMAN (version 4.13) and the NCBI databases (<http://www.ncbi.nlm.nih.gov>). The *C. saccharobutylicum gltAB* region was assigned GenBank accession number AF082880.

Cloning of regions adjacent to *glnA-nitR* in the *C. saccharobutylicum* genome. The *C. saccharobutylicum glnA* gene had been previously cloned on plasmid pHZ200 (Fig. 1) (Usdin *et al.*, 1986). The Bluescript pSK vector (Stratagene) was used for subcloning and sequencing. Plasmids pHS4 and pHS5 were isolated consecutively by chromosome walking, using a 0.38 kb *HindIII* probe from pHZ200 to identify pHS4, and a 0.67 kb *PvuII-XbaI* pHS4 fragment to detect the 3.5 kb *PvuII* fragment cloned in pHS5. For complementation of GOGAT activity in *E. coli*, the 3.5 kb *PvuII* fragment from pHS5 encoding the *gltB* gene was subcloned into pSK and pEcoR251 (Zappe *et al.*, 1986) to give pHS6 and pHS7. Plasmid pHS9 was constructed by first reconstituting the *gltA* gene by cloning the 3.8 kb *XbaI* fragment from pHS4 into the *XbaI* site of pHZ200, and then subcloning the entire gene on a *EcoRV-NotI* fragment into the unique *EcoRI* site of pACYC184 (Rose, 1988) after blunting the *NotI* and *EcoRI* sites (Fig. 1).

RNA preparation and northern hybridization analysis. Total mRNA was extracted from *C. saccharobutylicum* during early and late exponential phase as described by Aiba *et al.* (1981). Northern and dot blots were performed (Sambrook & Russell, 2001) using the DIG Labelling and Detection kit (Roche). The internal probes from the *glnA* and *nitR* genes were prepared as above, and probes for *gltA* and *gltB* were the 0.79 kb *EcoRV-XbaI* and 1.0 kb internal *XbaI* fragments respectively (Fig. 1). RNA dotblots were quantified using a densitometer and the software program GelTrak (D. Maeder, University of Cape Town). The detection and hybridization data for each probe were collected in triplicate.

GOGAT, GDH and GS enzyme activities. Cell-free extracts were prepared under strictly anaerobic conditions. Cells (~300 mg) were washed in 60 ml 20 mM KH₂PO₄/K₂HPO₄ buffer, pH 6.5,

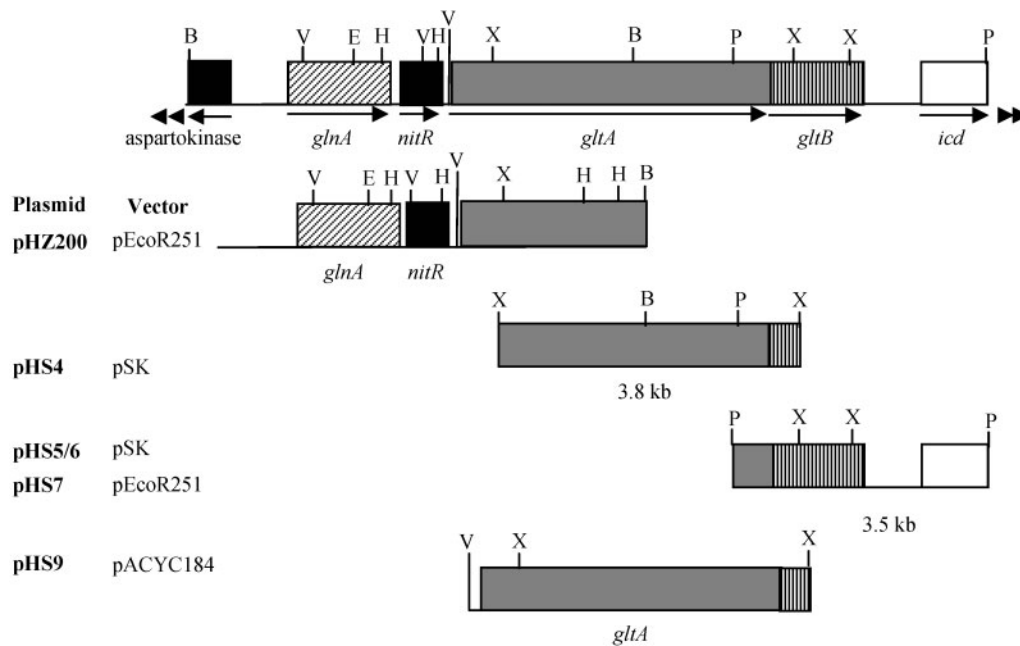


Fig. 1. Construction of plasmids containing the genomic regions adjacent to *glnA* and *nitR* from *C. saccharobutylicum* NCP262. The vector used was pSK, pEcoR251 or pACYC184, and the relative positions of the identified genes are shown with their transcriptional polarities below (not to scale). Restriction enzyme sites used for cloning were: B, *Bgl*II; E, *Eco*RI; V, *Eco*RV; H, *Hind*III; P, *Pvu*II; X, *Xba*I.

resuspended in 6 ml buffer and disrupted in a French pressure cell. The lysate was collected in a sealed Hungate tube on ice, and transferred back into the anaerobic cabinet for clarification and assays. Protein concentration was determined by the Bio-Rad assay system. GOGAT activity was determined spectrophotometrically by measuring the rate of NAD(P)H oxidation as described by Meister (1985), and was monitored in the anaerobic glove cabinet as it was highly sensitive to oxygen. Specific activity was expressed as μmol NADH oxidized per min per mg protein. Assimilatory GDH assays were essentially as in the GOGAT assay, with 100 mM NH_4Cl and 10 mM 2-oxoglutarate replacing glutamine. GS activities were measured by the γ -glutamyl transferase assay described by Shapiro & Stadtman (1968) and specific activity was expressed as μmol γ -glutamyl hydroxamate produced per min per mg protein.

RESULTS

The effect of nitrogen source on the growth of *C. saccharobutylicum*

In order to study the regulation of the key enzymes of nitrogen assimilation in *C. saccharobutylicum*, it was necessary to specify nitrogen-rich and nitrogen-limiting environments for this strain. The growth of both spore and cell-line cultures was monitored in a defined medium (GSMM) containing different nitrogen sources. Growth was directly proportional to the concentration of organic nitrogen added in the form of Casamino acids (Fig. 2a), with growth in 0.2% (w/v) Casamino acids being equivalent to that in complete medium (CBM) culture

(results not shown). Casamino acids could not be substituted by glutamine or glutamate, unlike in *B. subtilis*, where glutamine is a preferred source of nitrogen (Fisher & Sonenshein, 1991). Growth with 0.2% ammonium acetate as the sole nitrogen source was considerably inhibited and was equivalent to that with 0.01% Casamino acids (data not shown). Furthermore, the presence of ammonium acetate had a retarding effect on the germination and growth of the spore line cultures in the presence of 0.2% (w/v) Casamino acids (Fig. 2b). This effect was also observed with alternative sources of ammonia including NH_4Cl , NH_4NO_3 and $(\text{NH}_4)_2\text{SO}_4$ (data not shown).

GS, GOGAT and GDH activity during growth in different nitrogen conditions

Measurements of GS activities of steady-state *C. saccharobutylicum* cultures grown in GSMM with different nitrogen sources (Table 1), confirmed that 0.2% (w/v) Casamino acids is representative of nitrogen-rich growth conditions, with the most repressed levels of GS activity (referred to as non-inducing media). While the presence of inorganic nitrogen (ammonium acetate) had little effect on the levels of GS in the cells, GS activity was unexpectedly elevated early in the growth cycle in the presence of its product glutamine (Table 1). Thus, 0.025% (w/v) Casamino acids containing 0.15% (w/v) glutamine was selected to represent nitrogen-limiting conditions since GS activity was significantly induced in this medium (inducing

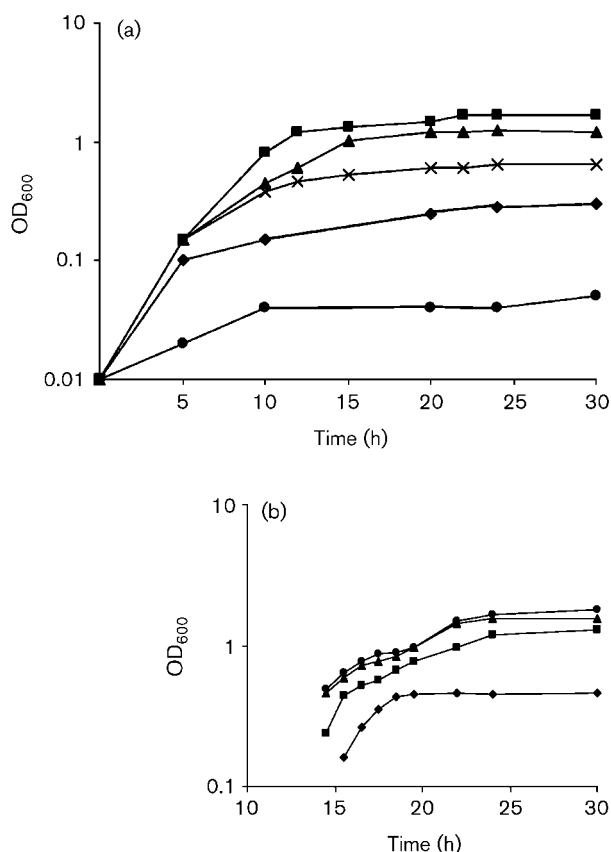


Fig. 2. Effect of organic and inorganic nitrogen sources on the growth of *C. saccharobutylicum* NCP262. (a) Growth profiles of cell-line cultures grown in GSMM containing 1% (w/v) glucose and various concentrations of Casamino acids (w/v): 0.2% (■), 0.1% (▲), 0.05% (×), 0.01% (◆) and none (●). (b) Growth profiles for spore-germinated cultures in GSMM containing 2% (w/v) glucose, 0.2% (w/v) Casamino acids, and various concentrations of ammonium acetate (w/v): 0.4% (◆), 0.2% (■), 0.1% (▲) and 0.05% (●). Growth curves are representative of three independent experiments for each growth condition.

media). These induced and non-induced trends were most pronounced at early exponential growth phase (OD_{600} 0.3) with levels differing as much as 9.2-fold (Table 1, Fig. 3b). Interestingly, growth of *C. saccharobutylicum* in either the inducing or non-inducing media with various concentrations of glutamate (0.1%, 0.5% or 1%) had no effect on either GOGAT or GS activity (data not shown).

GOGAT activity was also regulated by the nitrogen source, and both GS and GOGAT activities measured from the same culture were significantly induced or repressed by the same nitrogen conditions (Fig. 3). At early exponential growth phase (OD_{600} 0.3), there was a 5.6-fold difference in GOGAT activity between the inducing [$111 \mu\text{mol NADH oxidized min}^{-1} (\text{mg protein})^{-1}$] and non-inducing growth media [$20 \mu\text{mol NADH oxidized min}^{-1} (\text{mg protein})^{-1}$]. Similarly, a 6.2-fold increase was measured in GS activity in

Table 1. GS activity determined for spore-germinated cultures of *C. saccharobutylicum* NCP262 grown in GSMM containing 2% (w/v) glucose and various combinations of nitrogen sources, at early (OD_{600} 0.3) and late (OD_{600} 0.5) exponential growth phase

Each sample was assayed in duplicate. GS activity is expressed as $\mu\text{mol } \gamma\text{-glutamyl hydroxymate produced min}^{-1} (\text{mg}^{-1} \text{ protein})^{-1}$.

Nitrogen source*	Growth stage (OD_{600})	Sample size	Mean GS activity ($\pm$ SD)
CBM	0.3	4	0.09 ($\pm$ 0.01)
0.2% Casaa	0.3	8	0.13 ($\pm$ 0.02)
	0.5	3	0.06 ($\pm$ 0.02)
0.2% Casaa + 0.2% NH_4OAc	0.3	4	0.16 ($\pm$ 0.05)
	0.5	4	0.19 ($\pm$ 0.02)
0.05% Casaa	0.3	2	0.36 ($\pm$ 0.02)
	0.5	2	0.52 ($\pm$ 0.02)
0.05% Casaa + 0.2% NH_4OAc + 0.15% Gln	0.3	3	0.54 ($\pm$ 0.07)
	0.5	2	0.38 ($\pm$ 0.07)
0.05% Casaa + 0.15% Gln	0.3	10	0.54 ($\pm$ 0.06)
	0.5	6	0.34 ($\pm$ 0.11)
0.025% Casaa + 0.15% Gln	0.3	10	0.81 ($\pm$ 0.10)

*Casaa, Casamino acids; Gln, glutamine; NH_4OAc , ammonium acetate. Percentages are (w/v).

inducing media [$1.2 \mu\text{mol } \gamma\text{-glutamyl hydroxymate produced min}^{-1} (\text{mg protein})^{-1}$] compared to non-inducing media [$0.2 \mu\text{mol } \gamma\text{-glutamyl hydroxymate produced min}^{-1} (\text{mg protein})^{-1}$]. Both induced enzyme activities decreased towards late exponential phase, with GS levels dropping significantly (3.5-fold) between OD_{600} 0.6 and 0.8. This trend has also been observed in *B. subtilis* (Schreier, 1993), and suggests that neither enzyme is required at high levels during late stationary phase or sporulation. Neither of the enzyme activity levels fluctuated much throughout growth in non-inducing medium (Fig. 3). Assimilatory GDH activity could not be detected from either early or late-exponential phase cultures grown in either CBM, inducing or non-inducing media or in cultures restricted in carbon source (0.5 and 0.25%, w/v, glucose) and supplemented with ammonia (100 mM NH_4Cl).

Identification of genes flanking the *glnA* and *nitR* genes in *C. saccharobutylicum*

The cloning of the *glnA-nitR* genes on a 7.9 kb genomic fragment of *C. saccharobutylicum* was described previously (Usdin *et al.*, 1986). DNA sequence analysis of the region upstream from the *glnA* gene indicated a truncated aspartokinase gene. Downstream of the *glnA-nitR* genes, in the same orientation, are two genes encoding the large or α (*gltA*, 4554 bp) and small or β (*gltB*, 1473 bp) subunits of GOGAT. The initiation codon for *gltA* is

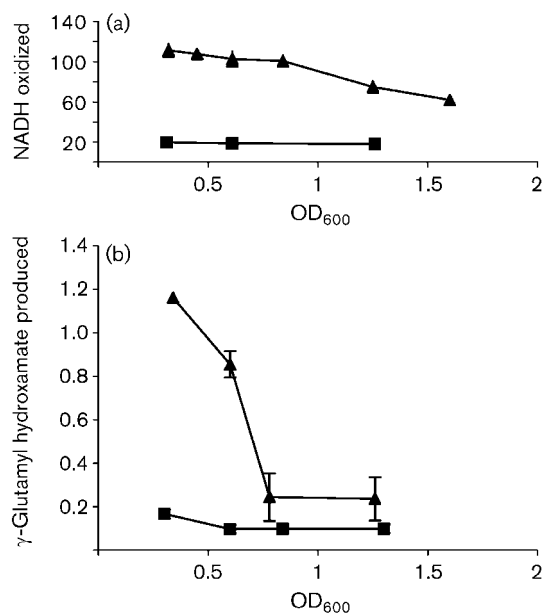


Fig. 3. (a) GOGAT activity [$\mu\text{mol NADH oxidized min}^{-1} (\text{mg protein})^{-1}$] and (b) GS activity [$\mu\text{mol } \gamma\text{-glutamyl hydroxamate produced (mg protein)}^{-1}$], measured from *C. saccharobutylicum* cultures grown in GSMM inducing medium (▲) and GSMM non-inducing medium (■), as a function of growth stage. Each result is the mean of three independent experiments performed in duplicate.

separated by 108 bp from the *nitR* stop codon. A truncated ORF was identified downstream from *gltB* encoding the amino-terminal region of isocitrate dehydrogenase, which catalyses the oxidative decarboxylation of isocitrate to form 2-oxoglutarate, the substrate for GOGAT.

The deduced amino acid sequences for *C. saccharobutylicum* GltA and GltB (1530 and 490 aa respectively) contain the highly conserved domains primarily identified by analysis of the *Azospirillum brasilense* enzyme subunits (Vanoni & Curti, 2005). The glutamine-binding and catalysis domain of PurF-type glutamine amidotransferases is conserved in the N-terminal domain of GltA, as is the cysteine cluster, characteristic of [3Fe-4S] containing enzymes. The NAD(P)H-binding domain in the β subunit contains the conserved residues, GXGXXG, characteristic of NADH binding (Vanoni & Curti, 2005). This is consistent with our GOGAT activity assays, where NADH, rather than NADPH, is the required coenzyme for *C. saccharobutylicum* GOGAT activity. This is unusual for bacterial GOGAT enzymes as the majority use NADPH, whereas use of NADH is typical of the eukaryotic enzymes (Vanoni & Curti, 2005).

Functional complementation of an *E. coli* *gltB* null mutant

In order to determine whether the *C. saccharobutylicum* *gltB* gene could functionally complement the mutation in

E. coli MX3004 (a glutamate auxotroph lacking the GOGAT β subunit), the *gltB* gene was cloned downstream of the lambda and *lacZ* promoters in pEcoR251 and pSK, yielding constructs pHS7 and pHS6, respectively (Fig. 1). Expression of *gltB* in *E. coli* MX3004, from either pHS6 or pHS7, failed to complement the *E. coli* α subunit, and did not restore growth of MX3004 on minimal medium containing ammonia as the sole source of nitrogen. However, co-transformation of pHS7 with pHS9, containing the *C. saccharobutylicum* *gltA* gene, did result in weak growth of the GOGAT mutant under anaerobic conditions only. RNA and protein analysis confirmed that both the *C. saccharobutylicum* *gltA* and *gltB* genes were effectively expressed in *E. coli* (data not shown).

Regulation of *glnA*, *nitR* and *gltAB* transcription

RNA was extracted from *C. saccharobutylicum* cultures grown to early and late exponential phase under the specified inducing and non-inducing conditions. Hybridization experiments showed that for all four genes, *glnA*, *nitR*, *gltA* and *gltB*, pronounced induction occurred under inducing conditions (Fig. 4, lanes 3 and 4) and repression was observed under non-inducing conditions (Fig. 4, lanes 1 and 2). The *glnA* and *nitR* probes detected a single RNA band of approximately 2.3 kb, resulting from the co-transcription of *glnA-nitR* under inducing conditions. Similarly, *gltA* and *gltB* form an operon, as the gene-specific probes hybridized to a common 6.2 kb band. Levels of mRNA were quantified from dot blots (results not shown). Signal intensities revealed that, at early exponential phase (OD₆₀₀ 0.30), there was a seven- to eightfold difference in the signals generated between the specified nitrogen-limiting vs nitrogen-rich growth media for both the *glnA* and *nitR* genes. The difference in the expression levels recorded for the *gltA* and *gltB* genes was also similar (5.5–6.0 fold), indicating co-transcription under all conditions. By OD₆₀₀ 0.7 there was a drop in the induced expression levels of *glnA* and *nitR* of approximately 1.6- and 1.7-fold respectively, whereas expression of the *gltA* and *gltB* genes remained relatively constant.

The regulatory regions of the nitrogen assimilation gene cluster

The genomic region corresponding to the nitrogen assimilation cluster of *C. beijerinckii* NCIMB 8052 (GenBank accession number DQ319904) had previously been identified by hybridization using the *glnA* and *nitR* genes of *C. saccharobutylicum* as probes (Quixley, 1999). The DNA sequence is identical to the recently released genome sequence of the same strain of *C. beijerinckii* (NZAALO01000002). The regions upstream of the nitrogen assimilation genes from *C. saccharobutylicum* and *C. beijerinckii* were aligned (Fig. 5), and show that these genes share common regulatory features. The two regions differ only in that *C. saccharobutylicum* has a longer intergenic region between *glnA* and *nitR*, while *C. beijerinckii* has an

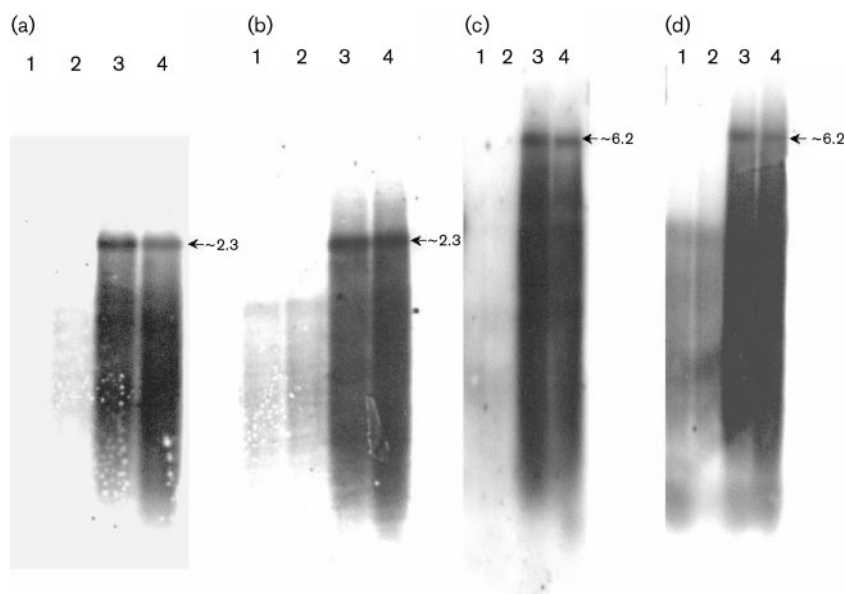


Fig. 4. Northern blots depicting transcriptional regulation of the *C. saccharobutylicum* *glnA* (a), *nitR* (b), *gltA* (c) and *gltB* (d) genes in response to nitrogen conditions. The RNA gene-specific probes were produced by PCR using probes internal to each gene. Lanes 1 and 2 each contain 30 µg RNA extracted from GSMM non-inducing medium at OD₆₀₀ 0.30 and 1.0, respectively. Lanes 3 and 4 each contain 30 µg RNA extracted from GSMM inducing media at OD₆₀₀ 0.30 and 0.7, respectively. Arrows highlight transcript sizes (kb).

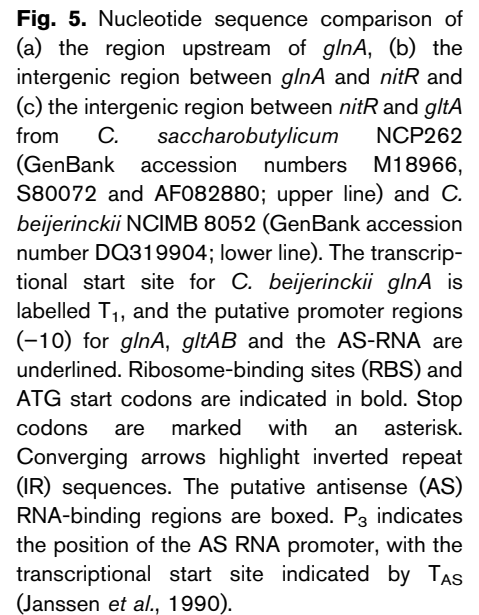
extra 66 bp between the *nitR* and *gltA* genes. The transcriptional initiation site upstream of the *glnA* gene, T1, and a putative promoter sequence with similarity to the clostridial extended consensus promoter sequences (Young *et al.*, 1989), has been identified previously in *C. beijerinckii* (Quixley, 1999). A number of regions of dyad symmetry with the potential to form stable stem-loop structures were identified in this leader region, IR1 ($\Delta G = -14.4$ kcal mol⁻¹; -60.2 kJ mol⁻¹) and IR2 ($\Delta G = -20.4$ kcal mol⁻¹; -85.4 kJ mol⁻¹; Fig. 5a). A similar stem-loop ($\Delta G = -12.1$ kcal mol⁻¹; -50.6 kJ mol⁻¹) was identified between the putative *gltA* promoter sequence and its structural gene (Fig. 5c). It is also noteworthy that the AS-RNA, which was shown to regulate *glnA* expression in *C. saccharobutylicum* by binding to a complementary region spanning the ribosome-binding site (RBS) and ATG start codons of the *glnA* gene with $\Delta G = -33.5$ kcal mol⁻¹ (-140.2 kJ mol⁻¹) (Fierro-Monti *et al.*, 1992; Fig. 5a), shares significant complementarity with the corresponding region of *gltA* ($\Delta G = -28.9$ kcal mol⁻¹; -120.9 kJ mol⁻¹) (Fig. 5c). Furthermore, the identical conserved clostridial RBS (Young *et al.*, 1989), 5'-AGGGGG-3', is present 7–8 bp upstream of the *glnA* and *gltA* initiation codons, respectively (Fig. 5a, c). The first two bases of the RBS for the *gltB* gene overlap the TAG termination codon for the *gltA* gene, suggesting that these two genes are translationally coupled. The presence of a transcriptional terminator ($\Delta G = -23.7$ kcal mol⁻¹; -99.2 kJ mol⁻¹), located 18–62 bp downstream of *gltB* (data not shown) is consistent with co-transcription of the *gltA* and *gltB* genes.

Bioinformatic analysis of the *nitR-gltAB* region

Comparison of the deduced amino acid sequences of the *gltA* and *gltB* genes from *C. saccharobutylicum* with those

from the recently sequenced genome of *C. beijerinckii* NCIMB 8052 (NCBI: NZAALO01000002) has shown identities of 87% and 83% for the α and β subunits respectively. In contrast, they showed only 49–53% identity to the predicted proteins from the other solvent-producing strain, *C. acetobutylicum* ATCC 824 (NCBI: NC003030). Phylogenetic analyses revealed that the GltA gene products from these two clostridia were most closely related to those from the lactic acid bacteria *Lactobacillus casei*, *Streptococcus mutans* and *Lactococcus lactis* (46–53% identity), while the nearest relatives of the deduced GltB proteins were from *Desulfotomaculum reducens* and *Synechocystis* (59% identity).

The *nitR* gene from *C. saccharobutylicum* encodes a response regulator (RR) protein belonging to the family pfam03861. All of these proteins have the conserved ANTAR domain, the RNA-binding domain found in transcription-antitermination regulatory proteins (Shu & Zhulin, 2002) and the REC domain, or signal-receiver domain, which is the phosphoacceptor region present in response regulators such as CheY (Galperin, 2006). The deduced NitR protein has significant similarity over its entire length to uncharacterized response regulators from *Streptomyces coelicolor* (NCBI: T35758) and *Mycobacterium tuberculosis* (NCBI: H70558), and to the NasT protein from *Azotobacter vinelandii* (Gutierrez *et al.*, 1995). NasT has been shown to be a positive regulator of the assimilatory nitrate/nitrite reductase operon, *nasAB*. In addition, the C-terminus of the predicted NitR protein shares similarity with C-terminal domains of several antiterminator regulatory proteins such as the aliphatic amidase regulator, AmiR, from *Pseudomonas aeruginosa* (Wilson *et al.*, 1993) and NasR, the positive regulator of the nitrite/nitrate reductase operon from *Klebsiella oxytoca* (Chai & Stewart, 1998).



Growth studies of *C. saccharobutylicum* indicated that organic nitrogen was essential for spore germination and was clearly the preferred type of nitrogen for growth and differentiation. The nitrogen status of the cells is reflected by the activities of both GS and GOGAT; however, GS levels were unexpectedly elevated in the presence of its product, glutamine. It is possible that glutamine is metabolized on entry, thereby raising the intracellular glutamate and ammonium concentrations and resulting in induction of the *glnA* and *gltAB* operons. In contrast, growth of *C. saccharobutylicum* in the presence of glutamate had no effect on either GOGAT or GS activity. Glutamate is known to repress the expression of the *glt* operons in *B. subtilis* and *E. coli* (Bohannon *et al.*, 1985; Castano *et al.*, 1988). In *C. pasteurianum*, an increase in the glutamine pool levels correlated with increased GOGAT activity (Kleiner & Fitzke 1979). There was no GDH activity detectable in *C. saccharobutylicum*, indicating that the energy-dependent GS/GOGAT pathway is the primary route for ammonia assimilation in the saccharolytic

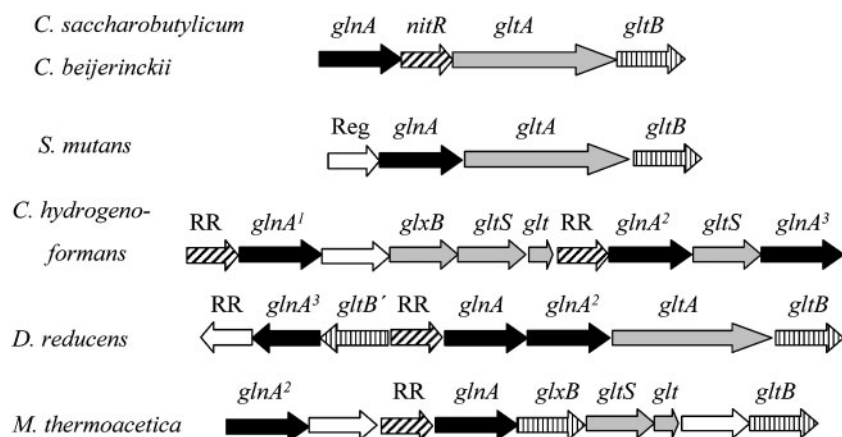


Fig. 6. Arrangement of genes in the nitrogen assimilation gene clusters from *C. saccharobutylicum* NCP262 (GenBank accession numbers: M18966, S80072 and AF082880) and *C. beijerinckii* NCIMB 8052 (DQ319904), *Streptococcus mutans* UA159 (NC004350), *Carboxydotherrmus hydrogenoformans* Z-2901 (NC007503), *Desulfotomaculum reducens* MI-1 (ZP01149008) and *Moorella thermoacetica* ATCC 39073 (NC007644). *glnA*, glutamine synthetase (black arrows); *nitR* or *RR*, ANTAR transcriptional regulator (hatched arrows); *gltA* (grey arrows) and *gltB* (striped arrows), α and β subunits of GOGAT; *Reg*, transcriptional regulator; *glxB*, glutamine amidotransferase class II; *gltS*, single-subunit GOGAT.

clostridia, as in *C. acetobutylicum* ATCC 824 (Amine *et al.*, 1990).

The genes encoding the α and β subunits of GOGAT are situated downstream of *glnA* in *C. saccharobutylicum*, and their function was confirmed by complementation of an *E. coli* GOGAT mutant, where the expression of both *Clostridium* subunit genes together enabled weak growth on minimal medium under anaerobic conditions only. The *Clostridium* β subunit alone was not able to functionally complement the *E. coli* α subunit, suggesting subunit incompatibility. Recent evidence has indicated that the α and β subunits of GOGAT enzymes form a tight complex which is stabilized by the [4Fe-4S] clusters at the interface of the two subunits (Vanoni & Curti, 2005). This specific interaction may not take place efficiently in *E. coli*. The native *C. saccharobutylicum* GOGAT enzyme was particularly sensitive to oxygen and requires a different co-factor to that from *E. coli*, factors which may contribute to the inefficient functioning of the *Clostridium* enzyme in this heterologous host.

The expression of the *glnA*, *gltA* and *gltB* genes showed that they were induced by nitrogen-limiting conditions and repressed by nitrogen-rich conditions. The *glnA* and *nitR* genes were co-transcribed as an operon on the same RNA transcript, as were *gltA* and *gltB*. Clearly, the changes in *glnA* and *gltAB* mRNA levels in relation to the nitrogen source were reflected in the corresponding levels of GS and GOGAT activities (Fig. 3), leading to the conclusion that these enzymes are similarly regulated by the same nitrogen conditions primarily at the level of transcription.

Several features of the nitrogen assimilation operons from *C. saccharobutylicum* and *C. beijerinckii* suggest that they are primarily controlled by an antitermination mechanism.

The position of the *nitR* gene between the *glnA* and the *gltAB* genes suggests that it has a key role in the regulation

of the operon. NitR represents a response regulator with the RNA-binding capability found in transcription anti-termination regulatory proteins (Shu & Zhulin, 2002; Galperin, 2006). The REC or signal-receiver domain would sense environmental nitrogen levels, while the ANTAR domain is proposed to act by binding to a stem-loop structure in the leader region of the RNA transcript, allowing transcriptional read-through into the structural genes. Molecular analysis of the nitrogen operons of *C. beijerinckii* and *C. saccharobutylicum* revealed a long leader mRNA transcript of ~200 bp upstream of the *glnA* genes (Quixley, 1999; Janssen *et al.*, 1990). The presence of several inverted repeat sequences with the potential to form intrinsic transcriptional terminators in these leader regions suggests that these may be the targets of the anti-terminator proteins. The identification of a transcriptional terminator between the putative *gltA* promoter and its structural gene suggests that the *gltAB* genes have a similar regulatory mechanism.

Based on these findings, we have proposed a model for nitrogen regulation in *C. saccharobutylicum* and *C. beijerinckii*. Under nitrogen-limiting conditions, the putative response regulator, NitR, is activated by a signal transduction mechanism and binds to a region of dyad symmetry present between the transcriptional start site and the *glnA* initiation codon, thus positively controlling *glnA* transcription via an antitermination mechanism. The *glnA* and *nitR* genes are transcribed together, with the transcript terminating at the inverted repeat sequence downstream of *nitR*. The activated NitR protein would regulate transcriptional read-through of this terminator as well so that both the *glnA-nitR* and the *gltAB* operons are simultaneously expressed.

Furthermore, under nitrogen-rich conditions, a 43 bp *glnA* AS-RNA (Fig. 5b), has been implicated in the down-regulation of GS expression by binding to a complementary sequence spanning the Shine-Dalgarno and start

codons of the *C. saccharobutylicum* *glnA* mRNA (Fig. 5a) (Fierro-Monti *et al.*, 1992). The AS-RNA also shows complementarity to a region spanning the Shine–Dalgarno and ATG start codon of *gltA* (Fig. 5c), providing the mechanism to downregulate the translation of both mRNA transcripts under the same conditions. The involvement of a post-transcriptional regulatory system is supported by the result that, at late exponential phase, the decrease in GS activity (3.5-fold) in nitrogen-limiting media was not reflected in the decrease in mRNA levels (1.6-fold).

The clostridia show great diversity in the genes involved in nitrogen assimilation. The clustering of the genes encoding GS and GOGAT, which is seen in *C. saccharobutylicum* and *C. beijerinckii*, is not found in *C. acetobutylicum* ATCC 824, where only *glnN*, encoding a GSIII enzyme, occurs. It is also not found in *Clostridium perfringens* (GenBank accession number NC003366) or *Clostridium tetani* (GenBank accession number NC004557), where the *glnA* genes are not adjacent to other nitrogen genes on the genomes. It is interesting that the nitrogen cluster in *S. mutans*, a member of the class *Bacilli* of the *Firmicutes*, includes the *glnA* and *gltAB* genes and is similar in arrangement to that of *C. saccharobutylicum* and *C. beijerinckii* (Fig. 6). This gene arrangement is not seen in the other streptococci. In addition, the regulator which is associated with the *S. mutans* *glnA* gene is very different to NitR and belongs to the MerR family. This suggests that the *S. mutans* *glnA*–*gltAB* gene cluster may have been acquired by horizontal gene transfer from the clostridia or vice versa, but that the regulator gene was sequestered from another source. The other species that show similar gene clustering to these clostridia also have response regulator proteins associated with them and belong to the *Clostridia* class of the *Firmicutes*. In these cases, the different gene arrangements could represent divergence from a common ancestor, with the acquisition of genes or modules at different times. The clustering of the genes encoding functional GS and GOGAT enzymes on the genomes of *C. saccharobutylicum* and *C. beijerinckii* and the co-ordinated regulation of these genes would appear to be the most simple and efficient means of ensuring that both enzymes are available at the same time. It may, however, also imply less metabolic flexibility and it will be interesting to know whether this novel regulatory model is also observed in other Gram-positive anaerobic bacteria.

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