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.

**INTRODUCTION**

There is renewed interest in the production of solvents and biofuels from renewable resources (Dürrre, 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...
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 glutB 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* MXX3004 (thi-1 gdh-1 pro-“ hutC glutD277::MudIHP13) (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 pH5 and pH5S were isolated consecutively by chromosome walking, using a 0.38 kb HindIII probe from pHZ200 to identify pH5S, and a 0.67 kb PvuII–XbaI pH5 fragment to detect the 3.5 kb PvuII fragment cloned in pH5S. For complementation of GOGAT activity in *E. coli*, the 3.5 kb PvuII fragment from pH5S encoding the gltB gene was subcloned into pSBK and pEcoR525 (Zappe *et al.*, 1986) to give pHS6 and pH5S. Plasmid pH5S was constructed by first reconstituting the gltA gene by cloning the 3.8 kb XbaI fragment from pH5S into the XhoI site of pHZ200, and then subcloning the entire gene on an 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,
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 mmol NADH oxidized per min per mg protein. Assimilatory GDH assays were essentially as in the GOGAT assay, with 100 mM NH₄Cl and 10 mM 2-oxoglutarate replacing glutamine. GS activities were measured by the c-glutamyl transferase assay described by Shapiro & Stadtman (1968) and specific activity was expressed as mmol c-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 NH₄Cl, NH₄NO₃ and (NH₄)₂SO₄ (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
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 Casamino acids (w/v): 0.2 % (■), 0.1 % (▲), 0.05 % (×), 0.01 % (●) and none (○). Growth curves are representative of three independent experiments for each growth condition.

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 μmol NADH oxidized min^{-1} (mg protein)^{-1}] and non-inducing growth media [20 μmol NADH oxidized min^{-1} (mg protein)^{-1}]. Similarly, a 6.2-fold increase was measured in GS activity in inducing media [1.2 μmol γ-glutamyl hydroxamate produced min^{-1} (mg protein)^{-1}] compared to non-inducing media [0.2 μmol γ-glutamyl hydroxamate produced min^{-1} (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_{4}Cl).

### 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

### 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

<table>
<thead>
<tr>
<th>Nitrogen source*</th>
<th>Growth stage (OD_{600})</th>
<th>Sample size</th>
<th>Mean GS activity (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBM</td>
<td>0.3</td>
<td>4</td>
<td>0.09 (±0.01)</td>
</tr>
<tr>
<td>0.2 % Casaa</td>
<td>0.3</td>
<td>8</td>
<td>0.13 (±0.02)</td>
</tr>
<tr>
<td>0.2 % Casaa + 0.2 %</td>
<td>0.5</td>
<td>3</td>
<td>0.06 (±0.02)</td>
</tr>
<tr>
<td>NH_{4}OAc</td>
<td>0.5</td>
<td>4</td>
<td>0.16 (±0.05)</td>
</tr>
<tr>
<td>0.05 % Casaa</td>
<td>0.5</td>
<td>4</td>
<td>0.19 (±0.02)</td>
</tr>
<tr>
<td>0.5 % Casaa</td>
<td>0.3</td>
<td>2</td>
<td>0.36 (±0.02)</td>
</tr>
<tr>
<td>0.05 % Casaa + 0.2 %</td>
<td>0.5</td>
<td>2</td>
<td>0.52 (±0.02)</td>
</tr>
<tr>
<td>NH_{4}OAc + 0.15 % Gln</td>
<td>0.3</td>
<td>3</td>
<td>0.54 (±0.07)</td>
</tr>
<tr>
<td>0.05 % Casaa + 0.15 % Gln</td>
<td>0.3</td>
<td>10</td>
<td>0.54 (±0.06)</td>
</tr>
<tr>
<td>0.005 % Casaa + 0.15 % Gln</td>
<td>0.5</td>
<td>6</td>
<td>0.34 (±0.11)</td>
</tr>
</tbody>
</table>

*Casaa, Casamino acids; Gln, glutamine; NH_{4}OAc, ammonium acetate. Percentages are (w/v).
enzymes. The NAD(P)H-binding domain in the cysteine cluster, characteristic of [3Fe-4S]

is conserved in the N-terminal domain of GltA, as is the catalysis domain of PurF-type glutamine amidotransferases.

The genomic region corresponding to the nitrogen assimilation gene 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 (NZAAL01000002). 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

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 gltA-nitR under inducing conditions. Similarly, gltA and gltB form an operon, as the genespecific 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 gltA 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 (NZAAL01000002). 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
extra 66 bp between the nitR and glnA 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 (ΔG = –14.4 kcal mol⁻¹; –60.2 kcal mol⁻¹ and IR2 (ΔG = –20.4 kcal mol⁻¹; –85.4 kcal mol⁻¹; Fig. 5a). A similar stem–loop (ΔG = –12.1 kcal mol⁻¹; –50.6 kcal mol⁻¹) was identified between the putative glnA promoter sequence and its structural gene (Fig. 5c). It is also noteworthy that the ASRNA, 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 ΔG = –33.5 kcal mol⁻¹ (–140.2 kcal mol⁻¹) (Fierro-Monti et al., 1992; Fig. 5a), shares significant complementarity with the corresponding region of glnA (ΔG = –28.9 kcal mol⁻¹; –120.9 kcal 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 glnA gene, suggesting that these two genes are translationally coupled. The presence of a transcriptional terminator (ΔG = –23.7 kcal mol⁻¹; –99.2 kcal 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 nitrate/nitrite reductase operon from Klebsiella oxytoca (Chai & Stewart, 1998).

**Fig. 4.** Northern blots depicting transcriptional regulation of the C. saccharobutylicum glnA (a), nitR (b), glnA (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).
Analysis of the genomic context of the close homologues of the *Clostridium glnA* and *gltAB* genes failed to identify other operons exactly like those of *C. saccharobutylicum* and *C. beijerinckii*. *C. acetobutylicum* 824 has no *glnA* gene, only a *glnN*, encoding a GSIII enzyme. The protein subunits of the GSIII family are substantially larger than those encoded by *glnA* genes, and the amino acid sequence shows only 9% sequence identity to the GSI family (van Rooyen et al., 2006). In *Streptococcus mutans* the *glnA* gene lies adjacent to the *gltAB* genes but they are associated with a regulator gene of the MerR family (Brown et al., 2003). In addition, three other *Firmicutes* show clustering of nitrogen assimilation genes: *Carboxydothermus hydrogenoformans*, *Desulfotomaculum reducens* and *Moorella thermoacetica*. In all three of these gene clusters, the *glnA* gene lies directly downstream of a regulator gene, which encodes a RR with both REC and ANTAR domains (Fig. 6). While *D. reducens* has the most similar gene arrangement to the clostridia, the other two species do not possess typical *gltAB* genes, and instead have various combinations of *gls*- and *gltB*-like genes, which may be involved in glutamate synthesis.

**DISCUSSION**

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
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 antitermination 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 antitermination 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.
codons of the *C. saccharobutylicum* ghN 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 glutA (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 ghN, 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 ghN 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 ghN and glutAB 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* ghN gene is very different to NitR and belongs to the MerR family. This suggests that the *S. mutans* ghN-gltAB gene cluster may have been acquired by horizontal gene transfer from the clostridia or vice versa, but that the regulator gene was sequenced 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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