The regulatory link between carbon and nitrogen metabolism in \textit{Bacillus subtilis}: regulation of the \textit{gltAB} operon by the catabolite control protein CcpA

Ingrid Wacker,† Holger Ludwig,† Irene Reif, Hans-Matti Blenccke, Christian Detsch and Jörg Stülke

Lehrstuhl für Mikrobiologie, Institut für Mikrobiologie, Biochemie und Genetik der Friedrich-Alexander-Universität Erlangen-Nürnberg, Staudtstr. 5, D-91058 Erlangen, Germany

\textit{Bacillus subtilis} assimilates ammonium by the concerted action of glutamine synthetase and glutamate synthase. The expression of the \textit{gltAB} operon encoding the latter enzyme is impaired in \textit{B. subtilis} ccpA mutant strains. CcpA is a pleiotropic transcriptional regulator that is the key factor in the regulation of carbon metabolism. However, in addition to their defect in catabolite repression ccpA mutants are unable to grow on minimal media with glucose and ammonium as the single sources of carbon and nitrogen, respectively. In this work, the expression of the \textit{gltAB} operon was analysed and its role in growth on minimal sugar/ammonium media was studied. Expression of \textit{gltAB} requires induction by glucose or other glycolytically catabolized carbon sources. In \textit{ccpA} mutants, \textit{gltAB} cannot be induced by glucose due to the low activity of the phosphotransferase sugar transport system in these mutants. A mutation that allowed phosphotransferase system activity in a \textit{ccpA} background simultaneously restored glucose induction of \textit{gltAB} and growth on glucose/ammonium medium. Moreover, artificial induction of the \textit{gltAB} operon in the \textit{ccpA} mutant allowed the mutant strain to grow on minimal medium with glucose and ammonium. It may be concluded that expression of the \textit{gltAB} operon depends on the accumulation of glycolytic intermediates which cannot occur in the \textit{ccpA} mutant. The lack of \textit{gltAB} induction is the bottleneck that prevents growth of the \textit{ccpA} mutant on glucose/ammonium media. The control of expression of the \textit{gltAB} operon by CcpA provides a major regulatory link between carbon and amino acid metabolism.

INTRODUCTION

\textit{Bacillus subtilis} utilizes glucose and glutamine as the preferred sources of carbon and nitrogen, respectively. If glucose or glutamine is present in the growth medium, the genes encoding enzymes involved in the utilization of secondary sources of carbon and nitrogen, respectively, are not expressed. This phenomenon is called catabolite repression.

The global control of carbon catabolism in \textit{B. subtilis} is exerted by a pleiotropic regulatory protein, CcpA. In the presence of glucose, CcpA can interact with regulatory sites in the control regions of regulated operons to either repress or activate transcription. DNA-binding activity of CcpA is triggered by interaction with a protein of the phosphotransferase system (PTS), HPr or its regulatory parologue Crh. In the presence of glucose, HPr and Crh are phosphorylated by a HPr kinase/phosphorylase (HPrK/P) on a regulatory seryl residue. HPr(Ser-P) and Crh(Ser-P) act as cofactors for CcpA (Deutscher \textit{et al.}, 1995, 2002; Galinier \textit{et al.}, 1997; Henkin, 1996; Stülke & Hillen, 2000). Recent proteome and transcriptome studies have demonstrated that about 250 and 85 genes are subject to CcpA-dependent repression and activation, respectively. Among the genes repressed by CcpA are those encoding enzymes required for the utilization of secondary carbon sources, but also genes of the Krebs citric acid cycle. The genes activated by CcpA include those required for overflow metabolism, glycolysis and the biosynthesis of certain amino acids (Blencke \textit{et al.}, 2003; Moreno \textit{et al.}, 2001; Tobisch \textit{et al.}, 1999; Yoshida \textit{et al.}, 2001).

Nitrogen metabolism is controlled by another transcription regulator, TnrA. In the absence of glutamine or ammonium, this protein activates transcription of genes encoding enzymes to utilize secondary nitrogen sources and represses expression of the \textit{glnA} gene and the \textit{gltAB} operon encoding enzymes of glutamine biosynthesis (Belitsky, 2002; Fisher & Débarbouillé, 2002). In the presence of repressing nitrogen
sources, i.e. glutamine or ammonium, the glutamine synthetase binds and thereby inactivates TnrA (Wray et al., 2001).

The two regulatory systems allow the bacteria to utilize sequentially the available sources of carbon and nitrogen, thus enabling them to optimize their metabolism. There are, however, also interactions between carbon and nitrogen regulation. Glycolysis is induced by glucose, but full induction occurs only if amino acids are available as well. Similarly, the Krebs citric acid cycle is synergistically repressed by glucose and glutamate (Ludwig et al., 2001; Rosenkrantz et al., 1985; Sonenshein, 2002). The molecular mechanisms that allow control of gene expression by both carbon and nitrogen sources have not yet been elucidated in *B. subtilis*.

*B. subtilis* ccpA mutants are defective in carbon catabolite repression. This is, however, not their only phenotype: they also exhibit a severe growth defect on minimal media (Lindner et al., 1994; Martin et al., 1989). ccpA mutants are also unable to grow with glucose and ammonium as single sources of carbon and nitrogen, respectively (Faires et al., 1999; Miwa et al., 1994; Wray et al., 1994). The observation that ccpA mutants require glutamate (or a source of it) as a source of nitrogen led us to propose that CcpA might be involved in the control of glutamate biosynthesis (Faires et al., 1999). However, while ccpA mutants grow with glucose, ammonium and glutamate, growth is still slower than observed with wild-type bacteria. This can be circumvented by the addition of methionine and the branched-chain amino acids to the growth medium. The ilv–leu operon encoding enzymes of branched-chain amino acid biosynthesis is not fully expressed in ccpA mutants. The reason for the methionine requirement has not yet been elucidated (Ludwig et al., 2002a).

CcpA can regulate transcription in different ways. As mentioned above, many genes are controlled by CcpA by binding to a catabolite-responsive element (cre) in the control region. However, there are many CcpA-dependent genes which do not possess any detectable cre target sites (Blencke et al., 2003; Moreno et al., 2001; Yoshida et al., 2001). A novel mechanism of gene regulation was recently discovered for the gapA operon, encoding enzymes of the triose phosphate interconversion part of glycolysis. This operon is induced by glucose and other sugars, but induction by glucose cannot occur in a ccpA mutant. The genetic evidence suggested that this effect is exerted in an indirect way (Fillinger et al., 2000; Ludwig et al., 2001). Detailed analyses revealed that ccpA mutants are defective in the transport of sugars by the PTS. This defect results from a strongly increased phosphorylation of HPr on the regulatory Ser-46 in the ccpA mutant which prevents participation of HPr in sugar transport and phosphorylation (Ludwig et al., 2002b). Mutations that prevent phosphorylation of HPr on Ser-46 result in the restoration of sugar transport and gapA operon expression. Thus, due to the defective transport of PTS sugars in ccpA mutants, the internal inducer of the operon cannot accumulate, resulting in lack of induction of the gapA operon. This novel mode of control of gene expression by CcpA was defined as class II regulation (Ludwig et al., 2002b).

Ammonium assimilation involves two enzymes in *B. subtilis*: the glutamine synthetase catalyses the formation of glutamine from glutamate and ammonium, and the glutamate synthase converts 2-oxoglutarate and glutamine to two molecules of glutamate. One of these molecules of glutamate can be recycled to glutamine while the second molecule is now available for anabolism. This reaction is the main link between carbon metabolism in the Krebs citric acid cycle and nitrogen metabolism since glutamate is the universal donor of amino groups (Belitsky, 2002). In contrast to *Escherichia coli* and other bacteria, the glutamate dehydrogenase does not contribute to glutamate biosynthesis in *B. subtilis*. It was proposed that this enzyme has a catabolic role in *B. subtilis* (Belitsky & Sonenshein, 1998).

Thus, the glutamate synthase encoded by the gltAB operon is essential for glutamate biosynthesis in this bacterium. Expression of the gltAB operon is repressed in the absence of ammonium by the pleiotropic regulator TnrA (Belitsky et al., 2000). In addition, the operon is under positive control of the transcriptional activator GltC (Bohannon & Sonenshein, 1989). The signal to which GltC responds is currently unknown. In addition, the gltAB operon is subject to control by the pleiotropic regulator CcpA. This control may link ammonium assimilation to the carbon and energy state of the cell (Faires et al., 1999).

In this study, we investigated the mechanism by which CcpA controls expression of the gltAB operon and the relation between expression of gltAB and the growth defect of ccpA mutants. As observed for the glycolytic gapA operon, gltAB belongs to the recently discovered class II of CcpA-dependent genes. Expression of the operon requires induction by any of a variety of sugars. Induction by PTS sugars is prevented in ccpA mutants, consistent with the idea that the PTS is inactive in these mutants (Ludwig et al., 2002b). The formation of an internal inducer may thus result from transport and metabolism of the sugars. Mutations that allow expression of the gltAB operon independent of CcpA result in a suppression of the growth defect of the ccpA mutants. Thus, the inefficient expression of the gltAB operon is the major bottleneck that limits growth of ccpA mutants.

**METHODS**

**Bacterial strains and growth conditions.** The *B. subtilis* strains used in this study are listed in Table 1. E. coli DH5α (Sambrook et al., 1989) was used for cloning experiments. *B. subtilis* was grown in C minimal medium (70 mM KH₂PO₄, 30 mM KH₂PO₄, 25 mM (NH₄)₂SO₄, 0.5 mM MgSO₄, 10 μM MnSO₄, 22 mg ferric ammonium citrate l⁻¹) supplemented with tryptophan (at 50 mg l⁻¹) (Faires et al., 1999). CSE medium is C minimal medium supplemented with sodium succinate (6 g l⁻¹) and potassium glutamate (8 g l⁻¹). C-Glc is C minimal medium supplemented with glucose
Table 1. B. subtilis strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source/reference*</th>
</tr>
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<tbody>
<tr>
<td>168</td>
<td>trpC2</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>BGW2</td>
<td>trpC2 lys-3 ccpA::Tn917erm</td>
<td>Krüger et al. (1993)</td>
</tr>
<tr>
<td>QB5407</td>
<td>trpC2 cpaA::Tn917spc</td>
<td>Faires et al. (1999)</td>
</tr>
<tr>
<td>GP302</td>
<td>trpC2 cpaA::Tn917erm</td>
<td>Ludwig et al. (2002b)</td>
</tr>
<tr>
<td>GP222</td>
<td>trpC2 p_{ymA}::gltA cat</td>
<td>pGP724→168</td>
</tr>
<tr>
<td>GP223</td>
<td>trpC2 cpaA::Tn917erm p_{ymA}::gltA cat</td>
<td>BGW2→GP222</td>
</tr>
<tr>
<td>GP335</td>
<td>trpC2 cpaA::Tn917erm pshHI</td>
<td>Ludwig et al. (2002b)</td>
</tr>
<tr>
<td>GP342</td>
<td>trpC2 amyE::(gltA–lacZ aphA3)</td>
<td>pGP526→168</td>
</tr>
<tr>
<td>GP351</td>
<td>trpC2 cpaA::Tn917spc amyE::(gltA–lacZ aphA3)</td>
<td>QB5407→GP342</td>
</tr>
</tbody>
</table>

*Arrows indicate construction by transformation.

(1 g l^{-1}), and CE-Glc is C minimal medium supplemented with potassium glutamate and glucose (8 g l^{-1} and 1 g l^{-1}, respectively). E. coli was grown in LB medium and transformants were selected on plates containing ampicillin (100 µg ml^{-1}). LB and SP (Kunst & Rapoport, 1995) plates were prepared by the addition of 17 g l^{-1} Bacto agar (Difco) to the medium.

**DNA manipulation.** Transformation of E. coli and plasmid DNA extraction were performed by standard procedures (Sambrook et al., 1989). Restriction enzymes, T4 DNA ligase and DNA polymerases were used as recommended by the manufacturers. DNA fragments were purified from agarose gels using the Nucleospin extract kit (Macherey and Nagel). Pfu DNA polymerase was used for the polymerase chain reaction (PCR) as recommended by the manufacturer. DNA sequences were determined using the dye-deoxy chain termination method (Sambrook et al., 1989). Chromosomal DNA of B. subtilis was isolated as described by Kunst & Rapoport (1995).

**Transformation and characterization of the phenotype.** B. subtilis was transformed with plasmid and chromosomal DNA according to the two-step protocol (Kunst & Rapoport, 1995). Transformants were selected on SP plates containing chloramphenicol (5 µg ml^{-1}), spectinomycin (100 µg ml^{-1}), kanamycin (5 µg ml^{-1}), or erythromycin plus lincomycin (1 and 10 µg ml^{-1}, respectively). Quantitative assays of lacZ expression in B. subtilis were performed with cell extracts using ONPG as the substrate (Kunst & Rapoport, 1995).

**Plasmid constructions.** A translational fusion of the gltAB promoter to a promoterless lacZ gene was constructed using the vector pAC7 (Weinrauch et al., 1991), which allows the introduction of the fusion into the amyE locus of B. subtilis. The 245 bp gltA promoter fragment (216 bp upstream to 29 bp downstream of the translational start codon) was amplified by PCR using primer pair IW1 (5’AACGCGGATCCACTTGCGGCCGATAAAATACGGC3’) and IW2 (5’AAAGGATCTGACCGCTTTGGCATTTGATTGTACGTC3’). The PCR product was digested by EcoRI and BamHI (the sites were underlined in the sequences) and ligated with pAC7 linearized with the same enzymes. The identity of the cloned insert was verified by sequencing and the resulting plasmid was pGP526.

**Construction of a strain allowing expression of the gltAB operon under control of the P_{ymA} promoter.** To express the gltAB operon under a controllable promoter plasmid pGP724 was constructed as follows (see Fig. 1). 520 bp PCR fragment (extending from 53 bp upstream to 467 bp downstream relative to the translational start of gltA) was generated by PCR using primers HMB55 (5’AACGCGGATCCGTGTATGATTITATGACGGG3’) and HMB56 (5’AAACCGGATCCACTTGCGGCCGATAAAATACGGC3’). The resulting PCR product was digested with BamHI and ligated into plasmid pX2 (Mogk et al., 1997) linearized with BamHI (see Fig. 1a). The identity of the cloned insert was verified by sequencing. B. subtilis 168 was transformed with the resulting plasmid pGP724 and transformants were selected on SP plates containing chloramphenicol and xylose (1·5%, w/v). The resulting strain GP222 was able to grow in C-Glc minimal medium only in the presence of xylose (1·5%, w/v) or glutamate (0·8%, w/v). Strain GP223, carrying the cpaA::Tn917 insertion in addition to the xylose-inducible gltAB operon, was constructed by transformation of GP222 with chromosomal DNA of BGW2. The chromosomal arrangement of the gltAB operon of these strains is shown in Fig. 1(b).

**Northern blot analysis.** Preparation of total RNA of B. subtilis and Northern blot analysis were carried out as described previously (Ludwig et al., 2001). The gltA Digoxigenin RNA probe was obtained by in vitro transcription with T7 RNA polymerase (Roche Diagnostics) using a PCR-generated fragment obtained with primer pair HL45 (5’CAAAGGCTCAAGGCTCTCTCA3’) and HL46 (5’CTAAAATCGAATCACTATAAGGGAATATATACCTGACGGGAAAC3’). The reverse primer contained a T7 RNA polymerase recognition sequence (underlined in HL46). In vitro RNA labelling, hybridization and signal detection were carried out according to the manufacturer’s instructions (DIG RNA labelling Kit and detection chemicals; Roche Diagnostics).

**RESULTS**

**Regulation of the gltAB operon by the carbon source**

Previous work demonstrated that expression of the gltAB operon is induced by ammonium and by glucose (Belitsky et al., 2000; Faires et al., 1999). While the ammonium-dependent regulation by the transcriptional regulator TnrA was studied in detail, the mechanism of induction by glucose has remained unknown. To analyse the regulation of the gltAB operon by different carbon sources, we assayed the expression of a translational fusion of the gltAB promoter region to a promoterless lacZ gene present in B. subtilis GP342. This strain was grown in CSE minimal medium in the presence of different carbon sources and β-galactosidase activities were determined (Table 2). While the expression of the operon was very low in CSE medium, a more than...
20-fold induction was observed in the presence of glucose. Similarly, fructose, glycerol and glucitol induced \textit{gltAB} expression. Thus, the expression of the \textit{gltAB} operon is induced by carbon sources metabolized via the glycolytic pathway.

The role of the transcriptional regulator CcpA in sugar induction of the \textit{gltAB} operon

It has previously been reported that expression of the \textit{gltAB} operon is impaired in \textit{ccpA} mutants. However, no potential \textit{cre} target site for binding of CcpA was detected in the \textit{gltAB} promoter region (Faires \textit{et al.}, 1999). To study the role of CcpA in the induction of the \textit{gltAB} operon in more detail, we analysed the expression of the \textit{gltA–lacZ} fusion in the \textit{ccpA} mutant strain GP351 (Table 2). While the \textit{ccpA} mutation did not affect the basal expression of the fusion in CSE medium, there was no induction of \textit{gltAB} expression in the presence of either glucose or fructose. In contrast, the \textit{ccpA} mutation had no effect on the induction of the operon by glycerol and glucitol. The induction profile of the \textit{gltAB} operon in the \textit{ccpA} mutant strain was very similar to the regulation of the glycolytic \textit{gapA} operon. In both cases, CcpA was required for induction by sugars transported by the PTS, but not for non-PTS carbohydrates.

The specific loss of induction by PTS sugars in the CcpA mutant suggested that the lack of PTS activity was the reason for the absence of expression of the \textit{gltAB} operon. It had already been demonstrated that the reduced PTS activity in \textit{ccpA} mutants is due to an excessive kinase activity of HPrK/P, which interferes with phosphorylation of HPr by Enzyme I of the PTS (Ludwig \textit{et al.}, 2002b). If this were the reason for the loss of glucose induction of \textit{gltAB} in the \textit{ccpA} mutant, we would expect that a mutation that prevents phosphorylation

\begin{table}[h]
\centering
\caption{Induction of the \textit{gltAB} operon by different sugars}
\begin{tabular}{|l|c|c|}
\hline
Addition to CSE & \textit{β}-Galactosidase activity* & \\
 & Wild-type & \textit{ccpA} \\
\hline
None & 7 & 11 \\
Glucose & 157 & 15 \\
Fructose & 139 & 14 \\
Glycerol & 80 & 71 \\
Glucitol & 92 & 62 \\
\hline
\end{tabular}
\footnote{\textit{β}-Galactosidase activity is expressed in units (mg protein)$^{-1}$. Representative results from two or three independent experiments are shown. The variance of the different sets of experiments did not exceed 20\%.}
\end{table}
of HPr by the HPr kinase would restore sugar transport and concomitant induction of the \textit{gltAB} operon. Such a mutation is the \textit{ptsH1} mutation, which affects the regulatory phosphorylation site in HPr. The \textit{ccpA ptsH1} double mutant strain is indeed capable of transporting glucose (Ludwig \textit{et al.}, 2002b). To address the involvement of CcpA and the PTS in the regulation of \textit{gltAB}, we analysed the transcription of the operon in a wild-type strain (\textit{B. subtilis} 168) and in its isogenic derivatives GP302 (\textit{ccpA}) and GP335 (\textit{ccpA ptsH1}). RNA was extracted from cells of the three strains grown in CSE minimal medium in the absence or presence of glucose and subjected to Northern blot analysis using a riboprobe specific for \textit{gltA} (Fig. 2). In the wild-type strain, a 6-1 kb transcript was detected for glucose-grown cells. This mRNA size corresponds to the bicistronic \textit{gltAB} operon encoding the two subunits of glutamate synthase. As observed with the \textit{lacZ} fusion, no \textit{gltAB} expression was detectable after growth of \textit{B. subtilis} 168 in CSE medium without glucose. In the \textit{ccpA} mutant, glucose did not induce the transcription of the \textit{gltAB} operon. In contrast, induction was restored in the \textit{ccpA ptsH1} double mutant strain GP335. This finding is in good agreement with an analysis at the transcriptome level (Blencke \textit{et al.}, 2003) and with the idea that the reduced sugar transport by the PTS is the limiting factor for induction of the \textit{gltAB} operon in the \textit{ccpA} mutant, and suggests that \textit{gltAB} is the second recognized member of class II of CcpA-regulated genes (see Discussion).

**Effect of the \textit{ptsH1} mutation on growth of the \textit{ccpA} mutant in minimal medium**

\textit{B. subtilis} is able to grow with glucose and ammonium as single sources of carbon and nitrogen, respectively. However, \textit{ccpA} mutant strains cannot grow in such a medium unless provided with glutamate or another source of organic nitrogen (Fig. 3; see also Faires \textit{et al.}, 1999; Ludwig & Stülke, 2001). If expression of the \textit{gltAB} operon were the growth-limiting factor in the \textit{ccpA} mutant, we would expect that the \textit{ptsH1} mutation would suppress the growth defect in addition to restoring glucose induction of \textit{gltAB}. Indeed, the double mutant strain GP335 was able to

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**Fig. 2.** Influence of a functional CcpA on expression of the \textit{gltAB} operon. For Northern blot analysis total RNA was isolated from \textit{B. subtilis} 168 (wild-type), GP302 (\textit{ccpA}) and GP335 (\textit{ccpA ptsH1}) grown in CSE minimal medium in the absence (–) or presence (+) of glucose (0.5%, w/v). RNA was separated by electrophoresis in a 0.8% formaldehyde agarose gel. After blotting, the nylon membrane was hybridized to a \textit{gltA}-specific DIG-labelled riboprobe. Five micrograms of RNA per lane was applied. The size of the transcript corresponding to the full-length \textit{gltAB} operon mRNA (6-1 kb) is indicated. The sizes of 16S rRNA and 23S rRNA are indicated by arrows.

**Fig. 3.** Suppression of the growth defect of a \textit{B. subtilis} \textit{ccpA} mutant strain by introduction of the \textit{ptsH1} allele. The growth of the wild-type strain 168, the \textit{ccpA} mutant strain GP302 and the \textit{ccpA ptsH1} double mutant GP335 was monitored by measuring the OD_{600}. Cultures were grown at 37 °C with vigorous agitation in C-Glc (●) and CE-Glc (○). See Methods for details of media composition.
grow in the absence of glutamate with glucose and ammonium as the only sources of carbon and nitrogen, respectively (Fig. 3). However, the growth rate of the double mutant was reduced as compared to the wild-type strain growing in the same medium. This might result from insufficient expression of the *ilv–leu* operon in the mutant (Ludwig *et al.*, 2001). Moreover, both the wild-type and the *ccpA ptsH* double mutant strains grew faster in the presence of glutamate than in the medium containing ammonium as the single nitrogen source (see Fig. 3). This may be due to the need to assimilate ammonium via glutamine synthetase and glutamate synthase, which requires the consumption of one mole of ATP per mole of assimilated ammonium (Belitsky, 2002).

**Growth of the ccpA mutant in minimal media in the presence of different carbon sources**

As shown above, *B. subtilis* ccpA mutants are not able to use ammonium as a nitrogen source if glucose is present as a carbon source. This may be due to the lack of glucose induction of *gltAB* expression in the ccpA mutant. So far, growth of a ccpA mutant with ammonium and sugars different from glucose as single carbon sources has not been reported. Therefore, we compared the growth of the *B. subtilis* wild-type strain 168 and its isogenic ccpA derivative QB5407 in the presence of different carbohydrates. As shown in Fig. 4, the wild-type strain grew in the presence of all substrates tested. This correlates with the induction of the *gltAB* operon (see Table 2). In contrast, no growth of the ccpA mutant QB5407 was possible if the PTS sugars glucose or fructose were used as carbon sources. However, QB5407 grew if provided with glycerol or glucitol. This is in good agreement with the fact that the latter two carbohydrates are not transported by the PTS and induced the expression of the *gltAB* operon even in the ccpA mutant (see Table 2).

**Effect of artificially induced expression of the gltAB operon on the growth of a ccpA mutant**

The results presented above strongly suggest that the lack of *gltAB* expression and the resulting deficiency in ammonium assimilation is the reason for the growth defect of the ccpA mutant. To prove this hypothesis, we placed the *gltAB* operon under the control of a xylose-inducible promoter. The functionality of this system was tested by a Northern blot analysis of *gltAB* expression in the wild-type strain 168 whereas xylose did not cause any induction. In contrast, a very strong expression of the *gltAB* operon was observed in strain GP222 in the presence of xylose. Thus, artificial induction of the *gltAB* operon was confirmed. The generation times of the relevant *B. subtilis* strains in ammonium-based minimal media containing (i) succinate, glutamate, glucose and xylose, (ii) glucose, or (iii) glucose and xylose are shown in Table 3. The

![Fig. 4. Identification of carbon sources that allow growth of a ccpA mutant strain with ammonia as the single nitrogen source. The growth of the wild-type strain 168 and the ccpA mutant QB5407 was monitored by measuring the OD$_{600}$. Cultures were grown at 37°C with vigorous agitation in C-glycerol (▲), C-glucitol (▼), C-fructose (○) and C-glucose (●). The final concentration of the different carbon sources was 1.0%. Note that the scaling for the two strains is different due to large differences in the generation times.](image-url)
DISCUSSION

CcpA is the major player in the regulation of carbon catabolism in *B. subtilis* and other Gram-positive bacteria. In agreement with this notion, *ccpA* mutants do not exhibit carbon catabolite repression of numerous catabolic genes and operons. While this phenotype of *ccpA* mutants is immediately obvious, the reason for the growth defect observed with *ccpA* mutants of any tested species in minimal media has long been a matter of debate. In this work, we provide unequivocal evidence that lack of expression of the *gltAB* operon is the major bottleneck preventing growth of *B. subtilis ccpA* mutants in minimal media with glucose and ammonium as single sources of carbon and nitrogen, respectively.

Two alternative explanations have been proposed to explain the glutamate auxotrophy of *ccpA* mutants: while Faires *et al.* (1999) argued in favour of an insufficient expression of the *gltAB* operon, Belitsky (2002) suggested that the glutamate pool would be low in *ccpA* mutants due to the loss of carbon catabolite repression of *rocG*, encoding glutamate dehydrogenase (Belitsky & Sonenshein, 1998, 1999). Three lines of evidence presented in this work are clearly in agreement with the former idea. First, the *gltAB* operon is expressed in the *ccpA* mutant if non-PTS carbohydrates are present. Expression of the operon correlates with the growth of the *ccpA* mutant in the presence of these substrates. Second, the *ptsH1* mutation suppresses both the loss of *gltAB* expression and the growth deficiency without restoring carbon catabolite repression (Blencke *et al.*, 2003). Thus, loss of catabolite repression of *rocG* cannot be the cause of glutamate auxotrophy of the *ccpA* mutant. Finally, independent expression of the *gltAB* operon in a *ccpA* mutant is sufficient to suppress the growth defect. We were not able to get this result in a previous study (Faires *et al.*, 1999). Detailed analyses revealed that the construct used in that analysis was not fully inducible (our unpublished results). However, the *p*<sub>xytA</sub> system used for artificial induction in this work has already proven to be very useful in previous studies (Ludwig *et al.*, 2002a; Mogk *et al.*, 1997). Thus, expression of the *gltAB* operon is necessary and sufficient to allow the *ccpA* mutant strain to grow on minimal media with glucose and ammonium.

Expression of the *gltAB* operon is controlled by three factors. First, in the absence of ammonium, the operon is repressed by the pleiotropic transcriptional regulator of nitrogen growth of the *ccpA* mutant in minimal medium with glucose and ammonium.

### Table 3. Growth rates of *B. subtilis ccpA* mutant strains expressing the *gltAB* operon from its own or a xylose-inducible promoter

Cells were grown at 37°C with vigorous agitation in C minimal medium supplemented with glutamate (E, 0–8 %, w/v), succinate (S, 0–8 %), glucose (Glc, 0–5 %) or xylose (Xyl, 1–5 %) as indicated. Growth was monitored by measuring the OD<sub>600</sub>.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Generation time (min)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>gltAB</em></td>
<td>CSE-Glc/Xyl C-Glc C-Glc/Xyl</td>
</tr>
<tr>
<td>168</td>
<td>Wild-type</td>
<td>43 ± 1 64 ± 3 59 ± 3</td>
</tr>
<tr>
<td>GP302</td>
<td>Wild-type</td>
<td>63 ± 3 NG NG</td>
</tr>
<tr>
<td>GP222</td>
<td><em>p</em>&lt;sub&gt;xytA&lt;/sub&gt;::<em>gltA cap</em></td>
<td>43 ± 5 NG 73 ± 7</td>
</tr>
<tr>
<td>GP223</td>
<td><em>p</em>&lt;sub&gt;xytA&lt;/sub&gt;::<em>gltA cap</em></td>
<td>58 ± 5 NG 77 ± 7</td>
</tr>
</tbody>
</table>

*Generation times were determined from the growth of at least three independent cultures under each condition and are shown as means ± SD. NG, No growth.*
metabolism in *B. subtilis*, TnrA (Belitsky et al., 2000). Second, glutamate causes a mild repression of the *gltAB* operon. Third, expression of the *gltAB* operon is induced by the presence of carbohydrates such as glucose, glycerol and glucitol. Catabolism of all these carbohydrates involves glyceraldehyde 3-phosphate, which can then be further catabolized via the lower part of glycolysis. It is, however, unknown by which mechanism(s) repression and induction by glutamate and sugars, respectively, are exerted. The positive regulator of the *gltAB* operon, GltC, is a candidate for either control (Bohannon & Sonenshein, 1989).

Induction of the operon by glucose and fructose but not by glucitol and glycerol requires a functional CcpA protein. A similar induction pattern was observed for the glycolytic *gapA* operon of *B. subtilis* (Ludwig et al., 2002b). Moreover, we were not able to detect a potential cre site in the control region of the *gltAB* operon that might serve as a target for regulation by CcpA. We considered, therefore, that CcpA might play a similar role in induction of *gltAB* as recently demonstrated for the *gapA* operon: *gapA* mutants are impaired in PTS sugar transport and phosphorylation due to an excessive kinase activity of HPr kinase/phosphatase. Therefore, internal inducers derived from the catabolism of these sugars cannot accumulate in *ccpA* mutants, resulting in loss of induction of gene expression. The PTS defect of *gapA* mutants can be suppressed by a *ptsH1* mutation, which prevents HPr phosphorylation by the HPr kinase. Indeed, the *ptsH1* mutation did also suppress both the deficient expression of the *gltAB* operon and the growth defect of the *ccpA* mutant. Thus, the *gltAB* operon is the second recognized member of the class II of CcpA-responsive genes in addition to the glycolytic *gapA* operon. In a previous study, we isolated spontaneous mutants that exhibited suppression of the growth defect of *ccpA* mutants. One of these mutations, *sgd-1*, restored expression of the *gltAB* operon (Faires et al., 1999). However, the *sgd-1* mutation has so far not been identified. With the finding that *ptsH1* causes exactly the same phenotype, this latter mutation can be regarded as the first genetically defined *sgd* mutation understood at the molecular level.

For efficient growth of bacteria, the different branches of metabolism need to be tightly coordinated. Recent work demonstrates that there are several regulatory interrelationships between carbon and nitrogen metabolism in *B. subtilis*. As shown in this work, ammonium assimilation is strongly dependent on the carbohydrate supply. Moreover, synthesis of methionine and the branched-chain amino acids also depend on a functional CcpA, although to a lower degree (Ludwig et al., 2002a). In turn, several important steps of carbon metabolism are co-regulated by signals from carbohydrate and amino acid metabolism: expression of the glycolytic *gapA* operon is only fully induced if the cells are provided with both a sugar and a good source of amino acids (Ludwig et al., 2001); on the other hand, several genes encoding enzymes of the Krebs citric acid cycle such as *citZ* and *citB* are synergistically repressed by glucose and glutamate (Rosenkranz et al., 1985). Interestingly, the specific regulators of *citB* and *gltAB*, CcpC and GltC, respectively, are both members of the LysR family of transcriptional regulators (Bohannon & Sonenshein, 1989; Jourlin-Castelli et al., 2000; Schell, 1993).

To better understand both the regulation of the *gltAB* operon and the interdependence between carbon and nitrogen metabolism it will be necessary to study the molecular details of the induction process by sugars. Work with this aim is in progress in our laboratory.

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