Insufficient Expression of the *ilv-leu* Operon Encoding Enzymes of Branched-Chain Amino Acid Biosynthesis Limits Growth of a *Bacillus subtilis* ccpA Mutant

Holger Ludwig, Christoph Meinken, Anastasija Matin, and Jörg Stülke*

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

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*Bacillus subtilis* ccpA mutant strains exhibit two distinct phenotypes: they are defective in catabolite repression, and their growth on minimal media is strongly impaired. This growth defect is largely due to a lack of expression of the *gltAB* operon. However, growth is impaired even in the presence of glutamate. Here, we demonstrate that the *ccpA* mutant strain needs methionine and the branched-chain amino acids for optimal growth. The control of expression of the *ilv-leu* operon by CcpA provides a novel regulatory link between carbon and amino acid metabolism.

*Bacillus subtilis* uses glucose and glutamine as preferred sources of carbon and nitrogen, respectively. While the control of both carbon and nitrogen metabolism has attracted much attention during the past years (for reviews, see references 2, 5, and 20), the regulatory interrelation between the two metabolic branches has been the subject of much less analysis. A key reaction in ammonia assimilation, the biosynthesis of glutamate, seems to be subject to double control by glutamate and glucose for *B. subtilis*. This regulation involves specific and general regulators, GltC and CcpA, respectively (1, 3).

CcpA was discovered as a factor mediating carbon catabolite repression of many genes in *B. subtilis* (2, 9, 20). Moreover, CcpA is required as a positive regulator for the expression of genes encoding enzymes of glycolysis, overflow metabolism, and ammonia assimilation (3, 4, 23). In the presence of glucose or other well-metabolizable carbon sources, CcpA can bind its target sites (catabolite responsive elements [cre]) in the control regions of the regulated genes and repress or activate transcription. To bind DNA, CcpA needs to form a complex with either of two cofactors, the HPr protein of the phosphotransferase system or its regulatory parologue, Crh (6, 7).

In addition to its role as a transcriptional regulator, CcpA is involved in growth control. On minimal media, *B. subtilis* ccpA mutant strains exhibit a severe growth defect (12, 16, 24). This defect is most obvious on minimal media with glucose and ammonia as single sources of carbon and nitrogen, respectively. This was attributed to the lack of expression of the *gltAB* operon encoding glutamate synthase in the *ccpA* mutant (3). However, the molecular details of this regulation are not yet understood.

In this work, we describe experiments aimed at the identification of CcpA-dependent cellular functions that result in the growth defect of *ccpA* mutants. Among the amino acids present in caseine hydrolysate, we identified methionine and the branched-chain amino acids as important for growth of *ccpA* mutant strains in minimal media. The requirement for branched-chain amino acids results from a decreased expression of the biosynthetic *ilv-leu* operon in the *ccpA* mutant and can be bypassed by expression of this operon from an inducible promoter.

Identification of a chemically defined medium which supports fast growth of a *ccpA* mutant strain. Severe growth defects of *B. subtilis* ccpA mutants were observed in minimal media. Even in the presence of glutamate and glucose, the growth rate of the *ccpA* mutant is lower than that of wild-type strains (12). We attempted therefore to identify the factor(s) that is required to allow a growth rate of the *ccpA* mutant comparable to that of wild-type strains. The wild-type *B. subtilis* strain Q7144 (7) and its isogenic ΔccpA457 derivative, GP300 (13), were grown in C minimal medium containing glucose (C-Glc) (15) in the absence or presence of glutamate (0.8%, wt/vol) and caseine hydrolysate (0.1%, wt/vol), respectively. The wild-type strain grew under all conditions tested (Fig. 1A). However, the addition of amino acids as a nitrogen source (CE-Glc, CE-Glc-CAA) resulted in faster growth than was observed with a medium containing ammonia as a single source of nitrogen (C-Glc). In contrast, the *ccpA* mutant strain GP300 did not grow in the presence of glucose as a single source of carbon and ammonia as a single source of nitrogen (Fig. 1B).

The addition of glutamate (CE-Glc) restored growth of the *ccpA* mutant strain, but not to the rate seen for the wild-type strain. The addition of caseine hydrolysate to CE-Glc medium resulted in a further increase of the growth rate (Fig. 1B). The generation times in CE-Glc containing CAA were determined to be 37 and 47 min for the wild-type and *ccpA* mutant strains, respectively. Thus, one or more components that are present in caseine hydrolysate enable the *ccpA* mutant to grow nearly as fast as the wild-type strain.

The main components of caseine hydrolysate are amino acids. As observed with caseine hydrolysate, a synthetic mixture of amino acids (14) allowed the *ccpA* mutant to grow as fast as the wild-type strain if added to CE-Glc medium (Fig. 1B). This finding had two implications: first, it demonstrated...
that the amino acids really were required for rapid growth of the ccpA mutant strain, and second, this was the first chemically defined medium in which a ccpA mutant did not exhibit any growth defect.

Identification of a minimal set of amino acids that is required to support rapid growth of a ccpA mutant strain. To identify individual amino acids that are important for growth of the ccpA mutant, we prepared five different substractive pools of amino acids in which two to five amino acids were omitted according to their biosynthetic pathways. The omission of amino acids of the aspartate family (aspartate, lysine, threonine, methionine, and isoleucine) or of branched-chain amino acids (valine, leucine, isoleucine, and alanine) resulted in slower growth than was observed with CE-Glc medium containing caseine hydrolysate (data not shown). Thus, one or more of the amino acids omitted in these two pools may be necessary for efficient growth of the ccpA mutant strain.

The amino acids that were omitted in the two pools are all derived from aspartate and pyruvate as biosynthetic precursors. Thus, the ccpA mutant could be defective in synthesizing these precursors or in the downstream biosynthetic pathway(s).

To distinguish between these possibilities, we tested the effect of adding the precursors (aspartate and pyruvate) or of all the amino acids omitted in the two substractive pools (alanine, valine, leucine, isoleucine, aspartate, lysine, threonine, and methionine) to CE-Glc medium. While the addition of pyruvate and aspartate had no effect on growth of the ccpA mutant GP300, the amino acid mixture was as effective in supporting growth as the complete synthetic mixture of amino acids (Fig. 2). These data indicate that the amino acid biosynthetic pathways are not fully active in the ccpA mutant strain.

To define the amino acids required for growth of the ccpA mutant more precisely, we omitted individual amino acids from the mixture described above. These experiments identified a minimal mix composed of valine, leucine, isoleucine, and methionine as being required and sufficient to allow rapid growth of GP300 (Fig. 2). Thus, the biosyntheses of the branched-chain amino acids and of methionine may be defective in the ccpA mutant strain GP300.

Regulation of the ilv-leu operon by CcpA. Three genetic loci are involved in the biosynthesis of branched-chain amino acids in B. subtilis, the ilv-leu operon, the ilvA gene encoding threonine dehydratase, and the ilvD gene encoding dihydroxy acid dehydratase. First, we analyzed transcription of the ilv-leu operon and the presumable involvement of CcpA in the regulation of expression of the operon. RNA was isolated from the B. subtilis wild-type strain 168 and its isogenic ccpA derivative, GP302 (15) grown in CSE minimal medium (3) with or without glucose and subjected to a Northern blot analysis using a riboprobe specific for ilvB, the first gene of the ilv-leu operon. Two transcripts were observed in the wild-type strain, an 8.6-kb transcript corresponding to a heptacistronic mRNA encompassing all genes of the ilv-leu operon and a 3.8-kb transcript covering the three promoter-proximal genes, ilvB, ilvN, and ilvC. The amounts of both transcripts were increased in cells...
Control of the activity of the promoters of genes and operons involved in branched-chain amino acid biosynthesis by glucose. The data presented above demonstrate that CcpA is necessary for full expression of the ilv-leu operon. Next, we wished to study whether this control is exerted at the level of transcription initiation of the ilv-leu operon. Moreover, we asked if expression of ilvA and ilvD would also be under positive control of CcpA. A transcriptional fusion of an 848-bp fragment from 28 bp upstream to 498 bp downstream of the translational start codon of the ilvA gene was constructed using the plasmid pAC6 (21). The expression of this ilvA-lacZ fusion was analyzed in the wild type (GP339) and a ccpA mutant (GP341, constructed by transformation of B. subtilis 168 with chromosomal DNA of QB5407 (3)) to yield pGP518. Strain GP325 carrying a disrupted ccpA gene was, however, not subject to any regulation by CcpA (our unpublished results).

Xylose-inducible expression of the ilv-leu operon in a ccpA mutant overcomes the requirement of branched-chain amino acids. Our results indicate that the ilv-leu operon might be the only genetic locus involved in branched-chain amino acid biosynthesis which depends on a functional CcpA for full expression. To test this hypothesis more rigorously, we constructed a strain which carries the ilv-leu operon under the control of a xylose-inducible promoter. Briefly, a 526-bp PCR fragment from 28 bp upstream to 498 bp downstream of the translational start codon of the ilvB gene was cloned into the plasmid pX2 (17) to yield pGP518. B. subtilis strain 168 was transformed with the plasmid pGP518, and the resulting strain, GP324, was able to grow only in CSE minimal medium in the presence of xylose (1.5%, wt/vol) or caseine hydrolysate (0.1%, wt/vol). Strain GP325 carrying a disrupted ccpA gene in addition to the xylose-inducible ilv-leu operon was constructed by transformation of GP324 with chromosomal DNA of QB5407 (3).

To test the consequences of artificial induction of the ilv-leu operon on the growth behavior of the ccpA mutant strain, we determined the generation times of the ccpA mutant strain GP325 in which the ilv-leu operon is under control of the xylose-inducible promoter. The isogenic parent strain, QB5407, served as a control. As described above, this ccpA mutant strain grew, albeit slowly, on CE-Glc minimal medium. The addition of caseine hydrolysate resulted in a drastic decrease of the gen-

 grown in the presence of glucose. In contrast, only basal levels that were not increased in the presence of glucose were detected in the ccpA mutant strain GP302 (Fig. 3).

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FIG. 3. Influence of a functional CcpA on expression of the ilv-leu operon. For Northern blot analysis, total RNA was isolated from B. subtilis 168 (wild type) and GP302 (ccpA) grown in CSE minimal medium in the absence (−) or presence (+) of glucose (0.5%, wt/vol). RNA was separated by electrophoresis in a 0.8% formaldehyde agarose gel. After blotting, the nylon membrane was hybridized to an ilvB-specific digoxigenin-labeled riboprobe. Five micrograms of RNA was applied per lane. Preparation of total RNA of B. subtilis and Northern blot analysis were carried out as described previously (15). The ilvB digoxigenin RNA probe was obtained by in vitro transcription with T7 RNA polymerase (Roche Diagnostics) using a PCR-generated fragment obtained with the primer pair HL61 (5′-AATGTAACACACACGATGA-3′) and HL62 (5′-CTAAATACGACTCATATAGGGA-3′). The reverse primer contained a T7 RNA polymerase recognition sequence (underlined in HL62). The sizes of the transcripts corresponding to the full-length ilv-leu operon mRNA (8.6 kb) and ilvB-lnc (3.8 kb) are indicated. Note that the probe cross-hybridized with the 16S and 23S rRNAs. The sizes of 16S rRNA and 23S rRNA are indicated by arrows.

FIG. 4. Influence of a ccpA mutation on the promoter activity of an ilvB-lacZ fusion. B. subtilis strains GP339 (wild type) and GP341 (ccpA) carrying an ilvB-lacZ transcriptional fusion were grown in CSE medium with (grey bars) or without (black bars) glucose (0.5%, wt/vol). The β-galactosidase activities were measured in extracts prepared from exponentially growing cells (optical density at 600 nm, 0.6 to 0.8) and are expressed in units per milligram of protein. The values shown were derived from three independent measurements. Standard deviations are indicated.
TABLE 1. Growth rates of *B. subtilis* ccpA mutant strains expressing the ilv-leu operon from its own or a xylose-inducible promoter

<table>
<thead>
<tr>
<th>Strain (genotype)</th>
<th>Amino acid addition(s)</th>
<th>Generation time (min)</th>
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<tbody>
<tr>
<td>QB5407 (ccpA)</td>
<td>None</td>
<td>155 ± 7</td>
</tr>
<tr>
<td></td>
<td>CAA</td>
<td>55 ± 3</td>
</tr>
<tr>
<td></td>
<td>Xylose</td>
<td>156 ± 12</td>
</tr>
<tr>
<td></td>
<td>Met + xylose</td>
<td>109 ± 1</td>
</tr>
<tr>
<td>GP325 (ccpA P_{gal-}ilv-leu)</td>
<td>Met</td>
<td>NG*</td>
</tr>
<tr>
<td></td>
<td>Met + CAA</td>
<td>55 ± 5</td>
</tr>
<tr>
<td></td>
<td>Met + xylose</td>
<td>47 ± 1</td>
</tr>
<tr>
<td></td>
<td>Xylose</td>
<td>68 ± 0</td>
</tr>
</tbody>
</table>

* a Cells were grown at 37°C under vigorous agitation in CE-Glc minimal medium supplemented with caseine hydrolysate (CAA) (0.1% wt/vol), methionine (met) (0.002% wt/vol), or xylose (1.5% wt/vol) as indicated. The growth was monitored by measuring the optical density at 600 nm.

* b The generation times were determined from the growth of at least three independent cultures under each condition.

* c NG, no growth.

The generation time (55 versus 155 min; see Table 1 and Fig. 1B). The addition of xylose had no effect on the growth of this strain. The addition of methionine resulted in an intermediate generation time (Table 1). Strain GP325 was unable to grow on CE-Glc medium supplemented with only methionine. A combined addition of methionine and caseine hydrolysate allowed rapid growth comparable to that of strain QB5407 in CE-Glc in the presence of caseine hydrolysate. Interestingly, a similar rapid growth comparable to that of strain QB5407 in CE-Glc in CE-Glc medium supplemented with only methionine. A combination of the ilv-leu operon from its own or a xylose-inducible promoter resulted in a cell able to produce large amounts of amino acids.

While the regulation of individual metabolic pathways has been intensively studied with different bacteria, not much is known about the coordination of different metabolic pathways. For *E. coli*, specific components of the phosphotransferase system and the Crp-cAMP complex provide links between carbon and nitrogen metabolism (19, 22). Moreover, sulfur availability was proposed to control cAMP synthesis and thereby expression of genes involved in carbon catabolism for *E. coli* (18). The work presented here indicates that there is a tight coupling of carbon and nitrogen metabolism for *B. subtilis* as well and that CcpA is involved in integrating the different metabolic branches to achieve well-balanced growth.

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