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CcpA-Dependent Regulation of *Bacillus subtilis* Glutamate Dehydrogenase Gene Expression

Boris R. Belitsky,* Hyun-Jin Kim, and Abraham L. Sonenshein

Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Massachusetts 02111

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The *Bacillus subtilis rocG* gene, encoding catabolic glutamate dehydrogenase, was found to be subject to direct CcpA-dependent glucose repression. The effect of CcpA required the presence of both the HPr and Crh proteins. The primary CcpA binding site was identified by mutational analysis and DNase I footprinting. In the absence of inducers of the Roc pathway, *rocG* was still expressed at a low level due to readthrough transcription. CcpA-dependent repression of *rocG* readthrough transcription proved to contribute to the slow growth rate of *B. subtilis* cells in glucose-glutamate medium. Increased readthrough expression of *rocG* was shown to be partially responsible for the growth defect of *ccpA* strains in glucose-ammonium medium.

Interconversions of α-ketoglutarate and glutamate are major links between carbon and nitrogen metabolism. These reactions are catalyzed by several enzymes: glutamate synthase [α-ketoglutarate + glutamine + NAD(P)H → 2× glutamate + NAD^+(P)], glutamate dehydrogenase (GlutDH) [α-ketoglutarate + NH_3 + NAD(P)H ⇄ glutamate + NAD^+(P)], and glutamate-dependent aminotransferases [glutamate + keto acid (or aldehyde) ⇄ α-ketoglutarate + amino acid].

In *Bacillus subtilis*, two genes code for GlutDH proteins. The *rocG* gene product is the major catabolic GlutDH, while *gudB* encodes an intrinsically inactive GlutDH (7). Spontaneous gain-of-function mutations in *gudB* generate a second catabolic GlutDH, GudB1 (7). No anabolic glutamate dehydrogenase is present in *B. subtilis*, and all de novo synthesis of glutamate is catalyzed by glutamate synthase (3).

The *B. subtilis rocG* gene is a member of the RocR regulon (5). RocR also controls the *rocABC* and *rocDEF* operons, whose products catalyze degradation of arginine to glutamate (9, 16, 17). All three *roc* transcription units have SigL-dependent promoters, require RocR and AhrC proteins for expression, and are induced by arginine, ornithine, or proline. The RocG-catalyzed reaction (glutamate + NAD^+ → α-ketoglutarate + NH_3 + NADH) can be viewed as the final step in the utilization of arginine, ornithine, and proline, providing rapidly metabolizable carbon- or nitrogen-containing compounds for biosynthesis.

Many *B. subtilis* genes involved in utilization of alternative carbon sources are subject to carbon catabolite repression mediated by the CcpA protein (8, 11, 25, 34). We show here that *rocG* expression is also subject to CcpA-dependent carbon catabolite repression. In the course of this work, we discovered that *rocG* is transcribed not only from its own SigL-dependent promoter but also by readthrough from an upstream gene. In fact, the known growth defect of *ccpA* mutants in glucose-ammonium medium (13, 30, 33) can be partially relieved by blocking readthrough transcription of *rocG*.

* Corresponding author. Mailing address: Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Phone: (617) 636-3618. Fax: (617) 636-0337. E-mail address: bbelit02@granite.tufts.edu.

MATERIALS AND METHODS

Bacterial strains and culture media. The *B. subtilis* strains used in this study are listed in Table 1 or described in the table footnotes. The media and growth conditions for *B. subtilis* and *Escherichia coli* strains were described previously (7). TSS minimal medium was supplemented with 0.5% glucose, 0.4% succinate, or other carbon and nitrogen sources supplied at 0.2%.

Strain BB1316 [*rocR*(T120I)] was created by replacement of the *rocD*::*aphA3* mutation in strain BB1236 by the wild-type *rocD* allele with chromosomal DNA from strain QB5626 carrying the *rocR*(T120I) mutation, which is very tightly linked to *rocD*. The $rocD^+$ *rocR*(T120I) transformants were selected as small colonies on TSS-ornithine plates, which are unable to support growth of the parental strain. The presence of the *rocR*(T120I) mutation in strain BB1316 was confirmed by introducing a *rocD-lacZ* fusion and analyzing its expression pattern in different media (17).

Strain BB1575 (*ptsH1*) was constructed by transforming strain SMY with chromosomal DNA from strain GM1222 carrying a plasmid integrated into the chromosome close to the *ptsH1* mutation. The $PtsH^-$ strains were identified among chloramphenicol-resistant transformants as slow-growing isolates on TSS-mannitol plates (12).

DNA manipulations and transformation. Methods for plasmid isolation, agarose and polyacrylamide gel electrophoresis, use of restriction and DNA-modification enzymes, DNA ligation, PCR, and electroporation of *E. coli* JM107 cells were as described by Sambrook et al. (28). Isolation of chromosomal DNA and transformation of *B. subtilis* cells by chromosomal or plasmid DNA were done as described previously (7).

Construction of the *rocGp1* mutant. A double substitution mutation (*rocGp1*) within the putative *cre* site of the *rocG* gene (Fig. 1) was created by site-directed mutagenesis by the method of Kunkel (4, 20) with the mutagenic oligonucleotide oBB73 (5′-GATTTTTTAA***AGGCCT***TACATTAC [altered nucleotides are in bold]), and pBB907 (7) as the template. The presence of the mutation in the resulting plasmid, pBB1048, was confirmed by showing susceptibility to cleavage by StuI (the italics in oBB73) and DNA sequencing of the entire *rocG* regulatory region.

pBB1048 was introduced into *B. subtilis* strain BB1271 (Δ*rocG*::*ble*) and neomycin-resistant transformants, arising from single-crossover homologous recombination events, were selected. Strain BB1744 (*rocGp1*) was isolated from one of the $RocG^+$ transformants as a spontaneous neomycin- and phleomycin-sensitive derivative in which the plasmid carrying the neomycin resistance gene and the Δ*rocG*::*ble* allele, determining resistance to phleomycin, had been excised from the chromosome and lost. The replacement of the chromosomal *rocG* gene by the *rocGp1* allele and excision of the plasmid in strain BB1744 were confirmed by detection of high GlutDH activity in glucose-ornithine minimal medium and by probing for the loss of vector sequences.

Construction of *rocGp1-lacZ* fusions. pBB1096, containing the *rocGp1-lacZ* fusion, is identical to pBB923 but for the presence of the *rocGp1* mutation from pBB1048 and was created in two steps as described earlier for pBB923 (5). To create pBB1049 containing a *DAS-rocGp1-lacZ* fusion with the downstream activating sequence (DAS) relocated upstream of the *rocG* promoter, the 0.71-kb BamHI-AgeI fragment of pBB996 containing the promoter part of the *DAS-*

TABLE 1. *B. subtilis* strains used

Strain	Genotype	Source or reference
SMY	Wild type	P. Schaeffer
BR151MA ccpA::spc	*ccpA*::[(Tn*917-lac*Z)::*spc*] *trp*C2 *lys*-3	T. Henkin
GM1222	*ptsH1 ykw*C::pJH101 (*'ykwC' cat*) *trpC2 sacB'-lac*Z	12
QB5618	*rocD*::*aphA3 trpC2* Δ*amyE*::Φ(*rocD'-lacZ cat*)	17
QB5626	*rocR*(T120I) *trpC2* Δ*amyE*::Φ(*rocD'-lac*Z *cat*)	17
QB7096	*crh*::*aphA3 trpC2*	27
SG82	*lacA*::*tet trpC2*	10
BB1179	*ccpA*::[(Tn*917-lac*Z)::*spc*)]	SMY × DNA (BR151MA *ccpA*::*spc*)
BB1236	*rocD*::*aphA3*	SMY × DNA (QB5618)
BB1264	*rocG*::pBB907 (*'yweA rocG rocA'*) *neo*	SMY × pBB907
BB1271	Δ*rocG*::*ble* Δ*gudB*::*tet*[a]	7
BB1316	*rocR*(T120I)	BB1236 × DNA (QB5626)
BB1434	*rocG*::pBB923 [Φ(*rocG'p*$^+$*-lac*Z *erm*)]	5
BB1512	Δ*amyE*::Φ(*DAS-rocG'p*$^+$*-lac*Z *erm*)	SMY × pBB996 (5)
BB1537	Δ*rocG*::*ble* Δ*gudB*::*tet ccpA*::[(Tn*917-lac*Z)::*spc*]	BB1271 × DNA (BB1179)
BB1575	*ptsH1 ykwC*::pJH101 (*'ykwC' cat*)	SMY × DNA (GM1222)
BB1597	*rocR*(T120I) *ccpA*::[(Tn*917-lac*Z)::*spc*]	BB1316 × DNA (BB1179)
BB1628	*yweA*::pBB1013 (*'yweA' neo*)	SMY × pBB1013
BB1744	*rocGp1* Δ*gudB*::*tet*	BB1271 × pBB1048
BB1770	*rocGp1 yweA*::pBB1013 (*'yweA' neo*) Δ*gudB*::*tet*	BB1744 × pBB1013
BB1792	*rocGp1 yweA*::pBB1013 (*'yweA' neo*) *rocR*(T120I)	BB1316 × DNA (1770)
BB2062	*rocG*::pBB1096 [Φ(*rocG'p1-lac*Z *erm*)]	SMY × pBB1096
BB2063	Δ*amyE*::Φ(*DAS-rocG'p1-lac*Z *erm*)	SMY × pBB1049
BB2163	*ccpA*::[(Tn*917-lac*Z)::*spc*] *yweA*::pBB1013 (*'yweA' neo*)	BB1628 × DNA (BB1179)

[a] The Δ*gudB*::*tet* null mutation in the gene coding for the inactive GudB-GlutDH was present in some strains and served to prevent spontaneous *gudB1* mutations from allowing expression of active GudB1-GlutDH (7).

rocGp$^+$*-lacZ* fusion (5) was replaced by a similar fragment of pBB1048. Both pBB1096 and pBB1049 were integrated into the chromosome either at the *amyE* locus by a double-crossover recombination event or at the *rocG* locus by a single-crossover recombination event as described previously (5).

Construction of pBB1013. Plasmid pBB907 (7), containing the entire *rocG* gene and the promoterless coding region of the upstream gene *yweA*, was integrated into the chromosome of *B. subtilis* strain SMY, creating strain BB1264. To clone DNA adjacent to the site of integration of pBB907, the chromosomal DNA of strain BB1264 was digested with SacI, self-ligated, and introduced by electroporation into cells of *E. coli* strain JM107 *pcnB80 zad*::Tn*10* (21). The isolated plasmid, pBB932, had a 4.04-kb insert of chromosomal DNA carrying most of the *spsK* gene and the entire *spsL*, *yweA*, and *rocG* genes. pBB932 was unstable in *E. coli pcnB*$^+$ cells, apparently due to toxicity of the *yweA* product.

The 2.33-kb EcoNI-XhoI fragment was excised from pBB932 to create pBB960. Plasmid pBB1013 containing the internal, 0.25-kb-long part of the *yweA* gene was constructed by removing the 1.46-kb SacI-HpaI fragment from pBB960.

Gel shift and DNase I footprinting experiments. His_6-CcpA was purified as described previously (19). The 259-bp fragments (positions −181 to + 78 with respect to the *rocG* initiation codon) containing the *rocGp*$^+$ or *rocGp1* region were amplified by PCR from pBB907 and pBB1048, respectively, with oBB96 (5′-GTTAATCGATAGATTTGTAGTC) and ^{32}P-labeled oBB56 (5′-CTTAAT GATTGTTTGGGTAGAC) as primers. DNA-CcpA complexes were formed and analyzed as described before (19).

DNA sequencing. Relevant parts of the *rocG* gene were sequenced by the dideoxy chain termination method of Sanger et al. (29) with vector- or *rocG*-specific oligonucleotides as primers and a Sequenase reagent kit (Amersham Life Science) as recommended by the manufacturer. Plasmid double-stranded DNA to be sequenced was purified with a QIAprep Spin miniprep kit (Qiagen). DNA and protein sequences were analyzed with the DNA Strider and Blast programs (1, 23) and the SubtiList web site (http://genolist.pasteur.fr/SubtiList/) (26).

Enzyme assays. β-Galactosidase activity was determined as described previously (4) and expressed in Miller units (24). GlutDH activity was assayed as

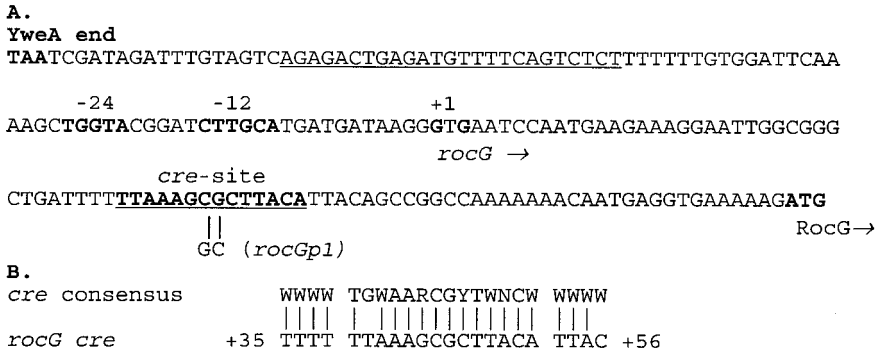

FIG. 1. (A) Sequence of the *rocG* regulatory region. The termination codon of *yweA*, the −12 and −24 promoter regions, the transcription start points, a likely initiation codon, and the core of a putative *cre* site of the *rocG* gene are in bold. The directions of transcription and translation are indicated by horizontal arrows. The dyad-symmetry sequence of a putative *yweA* transcriptional terminator and the region protected by CcpA in DNase I experiments are underlined. The sequence changes in the *rocGp1* mutant are indicated. (B) Comparison of *rocG cre* to the *cre* consensus sequence (36). Note that the second residue (G) of the core consensus sequence is not conserved in the *rocG cre* site.

TABLE 2. GlutDH activity in minimal medium[a]

Carbon source	Nitrogen source	GlutDH activity (U)				
		SMY wild type	BB1179 *ccpA*	BB1597 *ccpA* *rocR*(T1201)	BB1744 *rocGp1*	BB1792 *rocGp1* *rocR*(T120I)
Glucose	Ammonium	≤45	≤45	NG[b]	≤45	1,255
	Glutamate	≤45	≤45	1,052	≤45	1,005
	Ornithine	74	681	791	677	666
Ornithine	Ornithine	769	1,113	ND[c]	1,147	747
Glucose	Proline	76	62	883	63	967
Proline	Proline	246	177	ND	268	ND

[a] Cells were grown in minimal medium with the indicated carbon and nitrogen sources and assayed for GlutDH activity (7).
[b] NG, no growth under these conditions.
[c] ND, not determined.

described previously (7). One unit of GlutDH activity converts 1 μmol of NADH to NAD^+ per mg of protein per min. All numbers are averages of at least two experiments.

RESULTS

Glucose repression of *rocG* is dependent on CcpA. Previously we reported that the activity of *B. subtilis* GlutDH, encoded by the *rocG* gene, is highly increased in the absence of glucose if an inducer of the RocR pathway, e.g., arginine or ornithine, is present in the growth medium (7). Since the CcpA protein is a major regulator of carbon catabolite repression in *B. subtilis* (11, 18), we measured GlutDH specific activity in a *ccpA* mutant. Inactivation of the *ccpA* gene caused a full induction of RocG activity in cells grown in glucose-ornithine medium (Table 2, strains SMY and BB1179).

Expression of a transcriptional *rocG-lacZ* fusion, placed at the *rocG* locus, was also increased in a *ccpA* mutant in glucose-ornithine medium and was similar to the fusion activity seen in wild-type cells in the presence of ornithine as the sole carbon source (Table 3, strains BB1434 and BB1439). Such a fusion is expressed at a low level because its promoter is separated from the downstream activating sequence (DAS) required for *rocG* expression by the *lacZ* gene and plasmid sequences integrated at the *rocG* locus (5). A more active transcriptional fusion, in which the DAS region was relocated upstream of the *rocG* promoter (*DAS-rocG-lacZ*) (5), was regulated by CcpA in the same manner as RocG enzyme activity and the fusion at the *rocG* locus (Table 4, strains BB1512 and BB1521). Thus, CcpA regulates *rocG* expression at the transcriptional level, and this regulation is independent of the location of the DAS.

The *rocR*(T120I) allele, encoding a constitutively active version of the RocR protein, causes *rocG* expression to be largely independent of the presence of inducers (5, 17) but still subject to catabolite repression (Table 4, strain BB2131). In the *ccpA* *rocR*(T120I) double mutant, the activities of GlutDH and *rocG-lacZ* fusions were highly elevated not only in glucose-ornithine medium but also in glucose-glutamate medium. i.e., in the absence of inducers (Tables 2 to 4, strains BB1597, BB1470, and BB2139).

Role of auxiliary proteins in catabolite repression of *rocG*. The activity of CcpA as a transcriptional regulator in most cases depends on the presence of one or two additional proteins, HPr and Crh, encoded by the *ptsH* and *crh* genes, respectively (11). *rocG* expression was still repressed by glucose even if only one of these two proteins was active in the cells (Table 5, strains BB2025 and BB2026). Inactivation of both proteins in strain BB2027 (*ptsH1 crh*) led to derepression of the *DAS-rocG-lacZ* fusion in glucose-ornithine medium (Table 5). Therefore, to repress *rocG*, CcpA must interact with either Hpr or Crh.

Isolation and characterization of a *rocG cre* mutant. A putative CcpA binding site (*cre*), corresponding closely to pro-

TABLE 3. Activity of *rocG-lac*Z fusions[a]

Strain	Relevant genotype	Promoter region within the fusion	β-Galactosidase activity (U)					
			Medium with glucose plus:				Medium without glucose but with:	
			NH_4Cl	Glutamate	Proline	Ornithine	Proline	Ornithine
BB1434	Wild type	*rocGp*$^+$	0.39	0.47	0.49	0.52	2.3	11.3
BB1439	*ccpA*	*rocGp*$^+$	1.1	1.6	1.2	11.7	3.2	16.8
BB1470	*ccpA rocR*(T120I)	*rocGp*$^+$	NG[b]	6.2	7.1	14.2		
BB2155	*lacA*	*rocGp*$^+$	0.15	0.10	0.54			
BB2157	*lacA ccpA*	*rocGp*$^+$	0.54	0.32	18.3			
BB2062	Wild type	*rocGp1*	1.9	1.0	13.9	11.5		
BB2156	*lacA*	*rocGp1*	2.8	1.2	19.9			
BB2158	*lacA ccpA*	*rocGp1*	0.48	0.28	12.4			

[a] All strains are derivatives of BB1434 or BB2062 and contain a *rocG-lac*Z fusion integrated at the *rocG* locus. Cells were grown in minimal medium with the indicated carbon and nitrogen sources and assayed for β-galactosidase activity.
[b] NG, no growth under these conditions.

TABLE 4. Expression of *DAS-rocG-lacZ* fusions[a]

Strain	Relevant genotype	Promoter region within the fusion	β-Galactosidase activity (U)					
			Medium with glucose plus:				Medium without glucose but with:	
			NH_4Cl	Glutamate	Proline	Ornithine	Proline	Ornithine
BB1512	Wild type	*rocGp*$^+$	0.12	0.76	11.4[b]	11.9	35.1	233.9
BB1521	*ccpA*	*rocGp*$^+$	1.6	1.4	19.6	187.8	50.1	160.8
BB2131	*rocR*(T120I)	*rocGp*$^+$	3.6	13.5	14.6	9.6	138.7	228.8
BB2139	*ccpA* *rocR*(T120I)	*rocGp*$^+$	NG[c]	247.7	268.6	250.0	252.1	279.2
BB2063	Wild type	*rocGp1*	0.16	1.1	275.8	251.0	93.9	336.4
BB2064	*ccpA*	*rocGp1*	1.2	1.0	11.2	242.0		
BB2132	*rocR*(T120I)	*rocGp1*	56.5	166.0	245.1	200.0		

[a] Derivatives of strains BB1512 and BB2063 carrying *DAS-rocG-lacZ* fusions at the ectopic *amyE* locus (with the *rocG* DAS sequence positioned upstream of the fusion promoter) were grown in minimal medium with the indicated carbon and nitrogen sources and assayed for β-galactosidase activity.
[b] Low activity of the *rocG-lacZ* fusion (1 U) was detected during early exponential growth.
[c] NG, no growth under these conditions.

posed *cre* consensus sequences, was found about 40 bp downstream of the *rocG* transcription start point and 30 bp upstream of the initiation codon (Fig. 1). The two conserved central nucleotides of this sequence were altered by site-directed mutagenesis (Fig. 1), and the mutant *rocGp1* gene was substituted for the wild-type *rocG* allele in the *B. subtilis* chromosome as described in Materials and Methods. In contrast to wild-type cells, strain BB1744 (*rocGp1*) had high GlutDH activity in glucose-ornithine medium, similar to GlutDH activities found for the wild-type strain grown with ornithine as the sole carbon and nitrogen source and in the *ccpA* mutant grown in ornithine medium with or without glucose (Table 2). Similarly, *rocGp1-lacZ* fusions were highly expressed in ornithine medium even in the presence of glucose (Tables 3 and 4, strains BB2062 and BB2063). Thus, the *rocGp1* mutation alters a site required for CcpA-dependent glucose repression of *rocG*. As expected, the *rocGp1* promoter directed high GlutDH or *lacZ* fusion expression even in the absence of the RocR inducers if the constitutively active RocR(T120I) protein was present (Tables 2 and 4, strains BB1792 and BB2132).

CcpA binding to the *rocG* promoter. Purified CcpA bound efficiently to a 259-bp DNA fragment containing the putative wild-type *cre* site. CcpA formed several complexes with wild-type DNA, the number and relative abundance of which depended on the concentration of CcpA, indicating the presence of several binding sites or multimerization of the protein (Fig. 2, lanes 1 to 9). Unexpectedly, CcpA also bound to a similar fragment containing the *rocGp1* mutation, although the binding was about fourfold less efficient. Some CcpA-DNA complexes formed with mutant DNA had different electrophoretic mobilities than those formed with wild-type DNA, indicating altered stoichiometry or geometry of the DNA-CcpA interaction and confirming the presence of sites which can interact with CcpA independently of *cre* (Fig. 2, lanes 11 to 19).

In DNase I footprinting experiments, CcpA, at a concentration of 39 nM or more, protected the wild-type DNA sequence corresponding to positions +39 to +52 with respect to the *rocG* transcription start point (Fig. 3, lanes 1 to 5). This sequence coincides with the core of the putative *cre* site. No detectable CcpA binding to the *rocGp1* version of the same region was observed at any CcpA concentration used (Fig. 3, lanes 6 to 10), indicating that the *rocGp1* mutation decreased CcpA affinity for the *cre* region at least 16-fold. An additional DNA region within both wild-type and *rocGp1* fragments, which overlapped the *rocG* promoter and the upstream sequence, appeared to be partially protected at the highest CcpA concentration used (Fig. 3, lanes 5 and 10).

Contribution of RocG to the growth defect of the *ccpA* null mutant. *ccpA* mutants are known to have a growth defect in glucose-ammonium medium that is relieved by addition of

TABLE 5. Role of the HPr and Crh proteins in catabolite repression of the *rocG* gene[a]

Strain	Relevant genotype	β-Galactosidase activity (U) in medium containing:	
		Ornithine and glucose	Ornithine, no glucose
BB1521	*ccpA*	191.6	191.6
BB2025	*ptsH1*	7.0	
BB2026	*crh*	13.1	
BB2027	*ptsH1 crh*	242.1	

[a] Derivatives of strain BB1512 carrying the *DAS-rocGp*$^+$*-lacZ* fusion were grown in minimal medium with the indicated carbon and nitrogen sources and assayed for β-galactosidase activity.

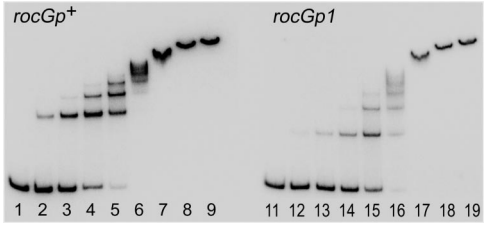

FIG. 2. Gel mobility shift analysis of the interaction between the *rocG* promoter and CcpA. Radioactively labeled 259-bp *rocGp*$^+$ (lanes 1 to 9) and *rocGp1* (lanes 11 to 19) promoter fragments were incubated with increasing concentrations of purified CcpA. The CcpA concentrations were 0 (lanes 1 and 11), 0.15 nM (lanes 2 and 12), 0.6 nM (lanes 3 and 13), 2.4 nM (lanes 4 and 14), 9.8 nM (lanes 5 and 15), 39 nM (lanes 6 and 16), 156 nM (lanes 7 and 17), 625 nM (lanes 8 and 18), and 2,500 nM (lanes 9 and 19).

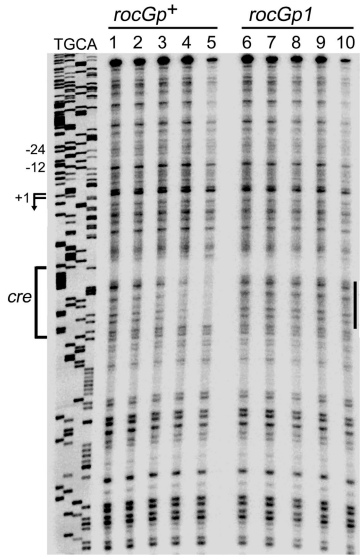

FIG. 3. DNase I footprinting analysis of CcpA binding to the *rocG* promoter. The 259-bp *rocGp*$^+$ (lanes 1 to 5) and *rocGp1* (lanes 6 to 10) promoter fragments, labeled on the template strand, were incubated with purified CcpA and then with DNase I. The sequence of the template strand of pBB907 (7) was determined with oBB56 as a primer. The apparent transcription start sites and the direction of *rocG* transcription are shown by the bent arrows, and the *cre* site is indicated by a bracket. The −12 and −24 promoter regions are shown. The protected *cre* area is indicated by a vertical line to the right of the gel lanes. Lanes 1 and 6, no CcpA; other lanes contained increasing concentrations of purified CcpA: 9.8 nM (lanes 2 and 7), 39 nM (lanes 3 and 8), 156 nM (lanes 4 and 9), and 625 nM (lanes 5 and 10).

glutamate or by overexpression of glutamate synthase (13, 30, 33). We were therefore prompted to analyze the possible contribution of RocG, an enzyme of glutamate catabolism, to this growth defect. In contrast to other reports, in our hands *ccpA* mutants grew, albeit significantly more slowly than the wild-type strain, on glucose-ammonium plates and in liquid medium (generation time of 100 to 200 min versus 60 min for the wild type). In addition, the lag time and the generation time in liquid medium varied from experiment to experiment, although no accumulation of revertants was detectable in most of our experiments. The growth defect of the *ccpA* null mutant was noticeably alleviated by introduction of the *rocG* null mutation, although the resulting strain did not grow in glucose-ammonium medium as fast as the wild-type strain did (generation time of about 80 min versus 60 min). On the other hand, introduction of the *rocR*(T120I) mutation, which increases *rocG* expression in the absence of inducers (Table 4, strain BB2131) (5, 17), completely abolished the ability of *ccpA* strains to grow in glucose-ammonium medium (Tables 2 to 4, strains BB1597, BB1470, and BB2139).

Liquid cultures of the *ccpA rocR*(T120I) double mutant accumulated revertant strains that were able to resume growth in glucose-ammonium medium. The new mutation in at least some of the revertants had the phenotype of the *rocG* null mutation and mapped to the *rocG* locus (data not shown); as expected, introduction of a characterized *rocG* null mutation restored the ability of the *ccpA rocR*(T120I) strain to grow in glucose-ammonium liquid medium (data not shown). We conclude that *rocG* expression, even at the low level seen in the absence of inducers, is partially responsible for the growth defect of *ccpA* mutants in glucose-ammonium medium.

Strains BB1316 [*rocR*(T120I)], BB1744 (*rocGp1*), and especially BB1792 [*rocGp1 rocR*(T120I)] also had growth defects in glucose-ammonium medium, presumably due to higher expression of GlutDH in these mutants; the defects were more pronounced on agar plates than in liquid medium (data not shown). Because the growth defect of these strains was less severe than the defect of the *ccpA* mutant and because a *rocG* null mutation only partially alleviated the *ccpA* growth defect, other factors must contribute to the poor growth of *ccpA* mutants (22, 30).

Readthrough expression of *rocG*. Though inactivation of the *rocG* gene greatly improved the ability of the *ccpA* mutant to grow in glucose-ammonium medium, inactivation of the *rocR* or *sigL* gene, both of which are required for expression of the *rocG* gene from its known promoter, did not affect the growth of the *ccpA* strain in this medium (data not shown). This result suggests that the low level of expression of *rocG* in glucose-ammonium medium initiates at a promoter that is independent of RocR and SigL.

The existence of such a promoter was demonstrated by integrating plasmid pBB1013, which carries an internal fragment of *yweA*, the gene immediately upstream of *rocG*, into the chromosome of a *B. subtilis ccpA* mutant (strain BB2163). Such a recombination event separates the *rocG* open reading frame and its SigL-dependent promoter from upstream chromosomal sequences. The presence of the intervening plasmid sequence relieved the growth defect of the *ccpA* mutant in glucose-ammonium medium to the same extent as did a *rocG* null mutation, indicating that *rocG* expression under these conditions initiated at an unidentified promoter located upstream of the integration point of pBB1013. Other, similar experiments showed that this promoter is located upstream of the *yweA* open reading frame. We suggest that transcription of *rocG* in glucose-ammonium medium is directed by the *yweA* promoter or the promoter of the upstream *sps* operon. Interestingly, this transcription would require readthrough of an apparent transcription terminator between *yweA* and *rocG* (Fig. 1).

The activity of RocG-GlutDH expressed from the upstream promoter was too low to detect by enzymatic assays. To quantitate the contribution of the upstream promoter to *rocG* expression, we tested the activity of *rocG-lacZ* fusions integrated at the *rocG* locus in strains carrying a mutation in *lacA*, which encodes an endogenous β-galactosidase (10). Expression of the *lacA* gene is subject to CcpA-dependent catabolite repression (25) and accounts for up to 1 Miller unit of β-galactosidase activity in minimal medium, obscuring expression of *lacZ* fusions with low activity. Under our growth conditions, the *lacA* strain with no *lacZ* fusion had 0.03 to 0.06 Miller units of β-galactosidase activity.

Very little activity of the *rocGp*$^+$*-lacZ* fusion was detected in glucose medium in the absence of RocR activators (e.g., ornithine); in contrast, expression of the *rocG-lacZ* fusions in the *ccpA* mutants in glucose-ammonium or glucose-glutamate medium was increased about threefold (Table 3, strains BB2155, BB2157, and BB2158), consistent with our suggestion that an increased level of *rocG* expression is partially responsible for the growth defect of this strain in glucose-ammonium medium.

This expression is apparently due to an increase in transcription readthrough which is normally blocked by binding of CcpA to the *cre* site. Unexpectedly, expression of the *rocGp1-lacZ* fusion with a mutation in the *rocG cre* site was severalfold higher in the *ccpA*$^+$ strain than *rocGp*$^+$*-lacZ* or *rocGp1-lacZ* expression in the *ccpA* mutant, although similar levels of expression were expected (Table 3, strains BB2156 and BB2158). Additional interactions between CcpA and the *rocG* regulatory region, other than with the *cre* site, could be responsible for this result.

Proline-mediated induction of *rocG*. Proline was shown to be an inducer of RocG but less effective than ornithine or arginine if glucose was absent from the medium (7). Accordingly, no significant relief of glucose repression was observed in glucose-proline medium with the *ccpA* mutant or the *rocGp1* strain containing a mutation in the CcpA binding site (Tables 2 to 4, strains BB1179, BB1744, BB1439, and BB1521). We suspect that this result reflects the fortuitous balancing of partial derepression of *rocG* and partial inactivation of TnrA, a global regulator of nitrogen metabolism genes (14, 15, 35), which may affect proline utilization. TnrA activity is abolished in cells grown in ammonium-containing medium (32). As expression of GlutDH in proline-containing medium is increased by inactivation of CcpA, by removal of glucose, or by the *rocGp1* mutation, degradation of glutamate derived from proline will generate ammonium under such conditions. As a consequence, TnrA activity and proline utilization will be reduced (6), preventing high-level induction of *rocG* and other *roc* genes. Similarly, it was previously shown (and we confirmed) that enzymes of the Roc pathway can be induced by proline but not by the combination of proline and ammonium (which inactivates TnrA) (2).

In accord with this model, when a *rocGp1-lacZ* fusion was placed at either the *rocG* or *amyE* locus in strains that were otherwise wild type (in which GlutDH synthesis was still subject to catabolite repression and glutamate degradation to ammonium was not significantly enhanced), *lacZ* was as highly expressed in glucose-proline medium as it was in glucose-ornithine medium (Tables 3 and 4, strains BB2062 and BB2063). Additionally, in the *ccpA rocR*(T120I) double mutant strain, in which *rocG* expression does not depend on proline uptake, high *rocG* expression was observed in all media tested, including the proline medium (Table 2 and 4, strains BB1597 and BB2139).

Glutamate utilization. The growth rate of *B. subtilis* cells in glucose-glutamate medium is severely limited by the low activity of RocG-GlutDH due to CcpA-mediated repression and inactivity of RocR under these conditions (7). The generation time in glutamate medium was decreased from 120 min for the wild-type strain to about 60 min if RocG-GlutDH activity was elevated by the presence of the constitutively active *rocR*(T120I) mutation or if total GlutDH activity was increased by the *gudB1* mutation (7).

Interestingly, the generation time of strain BB1744 (*rocGp1*) in glucose-glutamate medium also decreased to 85 min. In contrast, the *rocGp1* mutation had no effect on growth rate when the *rocG* gene was separated from the *yweA* promoter by insertion of plasmid DNA (strain BB1770). Therefore, even low-level readthrough transcription of *rocG* is sufficient for faster utilization of glutamate if catabolite repression of *rocG* is removed.

DISCUSSION

GlutDH, a ubiquitous metabolic enzyme, catalyzes a reversible reaction that can provide either the anabolic function of glutamate biosynthesis or the catabolic function of glutamate utilization. In *B. subtilis*, both RocG and the mutationally activated form of GudB (7) are exclusively catabolic enzymes. Neither RocG, even when fully derepressed by the *rocGp1* and *rocR*(T120I) mutations, nor GudB1 is able to support the growth of a glutamate synthase mutant in the absence of glutamate (our unpublished results).

In our previous work, we showed that *rocG*, like the *rocABC* and *rocDEF* operons, requires SigL-containing RNA polymerase, RocR, AhrC, and an enhancer-like activating sequence for expression (5, 15). Unlike the *rocABC* and *rocDEF* operons, which are highly expressed in minimal glucose-arginine medium (2, 9, 16) and are not significantly affected by the presence or absence of CcpA (data not shown), the *rocG* gene is subject to carbon catabolite repression mediated by CcpA acting at the *cre* site located between the transcription and translation start points. This differential regulation of the genes of the *roc* regulon probably reflects the different functional roles of the corresponding products. While the first three reactions of the Roc pathway liberate nitrogen groups from arginine and ornithine (15), RocG has a dual role, providing both ammonium as a nitrogen source and α-ketoglutarate as a carbon source. High activity of GlutDH is presumably not needed when rapidly metabolizable carbon sources are available and could lead to depletion of the pool of glutamate, a major cellular anion (31), if unchecked. As shown in the accompanying paper (6), high activity of GlutDH could drastically alter the regulation of nitrogen metabolism genes due to production of ammonium and consequent inactivation of a global regulator TnrA (14, 15, 35). In addition, GlutDH interferes with the activity of GltC, the activator of glutamate synthase (6). Interestingly, expression of *gudB* is also reduced in the presence of glucose, although the mechanism of this regulation remains unknown (7).

Binding of CcpA to the *rocG cre* site not only reduces the level of RocR-dependent *rocG* expression but also serves as a roadblock to prevent readthrough transcription from an upstream promoter. The latter promoter only contributes significantly to *rocG* expression in the absence of RocR activators, because induced RocR-dependent expression from the *rocG* promoter is much stronger than readthrough. Although readthrough transcription is only a minor contributor to *rocG* expression in wild-type cells when RocR is active, it permits multiple levels of expression. Thus, the *rocG* gene has four physiologically relevant modes of expression in minimal medium: no detectable expression in glucose medium in the absence of RocR activators; a very low level of expression in the absence of both glucose and the activators; a low to moderate level of expression in glucose medium in the presence of the activators; and a high level of expression in the absence of glucose and with the activators present. Interestingly, we recently found that a *cre* site located between the *ccpC* gene and

its upstream genes also regulates readthrough transcription of *ccpC* (19).

CcpA-dependent catabolite repression of GlutDH helps us to understand two previously unexplained phenomena. First, glutamate is known to be a poor nitrogen source for *B. subtilis* in glucose-containing medium. Since utilization of glutamate can be mediated by GlutDH activity, which is required to liberate ammonium for synthesis of glutamine, the negative effect of CcpA on *rocG* reduces the ability of cells to take full advantage of the potential nutritional value of glutamate. Second, *ccpA* mutants grow poorly in glucose-ammonium medium unless glutamate is added. The explanation in part seems to be that derepressed expression of *rocG* leads to degradation of endogenous glutamate. Additionally, higher RocG activity may interfere with glutamate synthesis due to inactivation of GltC (6). Reduced expression of glutamate synthase genes in *ccpA* mutants was documented previously (8, 13, 30).

The *rocG*, *rocABC*, and *rocDEF* genes have been shown in DNA array experiments to be regulated by glucose in a CcpA-dependent manner in cells growing in broth media (25, 34). In work to be published elsewhere, we found that this mode of regulation in broth media is unrelated to the *cre* site-dependent regulation of *rocG* described here for minimal media. Glucose catabolite repression of the *roc* genes in broth media is apparently due to poor uptake of arginine or proline or both.

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