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Regulation of the *Bacillus subtilis* Acetate Kinase Gene by CcpA

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The *Bacillus subtilis* gene encoding acetate kinase was identified on the basis of sequence similarity to the *Escherichia coli ackA* gene and to a second *E. coli* gene closely related to *ackA*. Insertional inactivation of this region of the *B. subtilis* chromosome resulted in the disappearance of acetate kinase enzyme activity in cell extracts. The *ackA* gene was mapped to a site close to the *ccpA* gene, at 263°. The transcriptional start site for *B. subtilis ackA* was located 90 bp upstream from the start of the coding region, and expression was increased by growth in the presence of excess glucose. Growth of the AckA$^-$ mutant was inhibited by glucose, suggesting that acetate kinase is important for excretion of excess carbohydrate. The stimulation of *ackA* expression by glucose was blocked in a CcpA$^-$ mutant, indicating that CcpA, which is required for glucose repression of certain carbon source utilization genes, including *amyE*, may also be involved in activation of carbon excretion pathways. Two sequences resembling the *amyO* operator site were identified upstream of the *ackA* promoter; removal of this region resulted in loss of glucose activation of *ackA* expression.

There are two pathways for interconversion of acetyl coenzyme A (acetyl-CoA) and acetate (Fig. 1). One pathway, which is mediated by acetyl-CoA synthetase, encoded by the *acsA* gene, operates via an acetadenylate intermediate. The *Bacillus subtilis acsA* gene has been identified (12). The second pathway, which utilizes an acetyl phosphate intermediate, is catalyzed by phosphotransacetylase, encoded by *pta*, and acetate kinase, encoded by the *ackA* gene. The *Escherichia coli ackA* gene has been cloned (22), and mutations in both *ackA* and *pta* have been characterized in *E. coli* and *Salmonella typhimurium* (3, 13, 18, 39). Most of these mutants were isolated by selection for fluoroacetate resistance; a similar selection scheme in *Bacillus stearothermophilus* did not yield mutants of this type (20).

Several lines of evidence suggest that the *pta-ack* pathway operates primarily in the acetyl-CoA-to-acetate direction, for acetate excretion. In *E. coli* and many anaerobic bacteria, conversion of acetyl phosphate to acetate by acetate kinase provides a major source of ATP during anaerobic growth (37). The K_m for acetate of acetate kinase in both *E. coli* and *B. subtilis* is approximately 300 mM (31, 36), while acetate can be utilized for growth at 25 mM, indicating that this enzyme is more likely to catalyze conversion of acetyl phosphate to acetate than the reverse reaction. Furthermore, *E. coli* and *S. typhimurium ackA* mutants retain a partial ability to grow on media containing acetate as the carbon source, suggesting the presence of alternate pathways for acetate utilization (3, 18). In *B. subtilis*, the *acsA* gene is required for acetate utilization (12).

B. subtilis excretes large amounts of acetate during growth in complex media containing excess carbohydrate (36). In *Bacillus cereus*, the addition of glucose to complex medium resulted in recovery of 95% of the carbon as extracellular pyruvate and acetate during vegetative growth in lightly buffered medium (14). Growth in the presence of excess glucose (e.g., 0.2%) also results in the accumulation of acetoin and butanediol, which are synthesized from pyruvate; synthesis of these neutral compounds prevents drastic acidification of the growth medium (36). Expression of the *alsS* gene, encoding acetolactate synthase, which carries out the first step in acetoin biosynthesis, is induced by the addition of glucose to the growth medium (29), and phosphotransacetylase enzyme activity is maximal in *B. subtilis* during vegetative growth in rich media (28). No information on acetate kinase expression has been reported. In this paper, we report the isolation and characterization of the *B. subtilis ackA* gene and demonstrate that *ackA* expression is stimulated by glucose. Glucose induction is dependent on the CcpA protein, which has previously been shown to act as a negative regulator of genes involved in utilization of secondary carbon sources (11, 16, 21) and which is required for acetoin biosynthesis (16, 43) and *alsS* expression (29). It therefore appears that CcpA acts as both a negative regulator of carbohydrate utilization genes and a positive regulator of genes involved in excretion of excess carbon.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. Bacteriophage M13 clones were propagated in *E. coli* JM103, and plasmid isolates were propagated in *E. coli* DH5α. *E. coli* strains were grown in LB medium (25). *B. subtilis* strains were grown in tryptose blood agar base (Difco), nutrient sporulation medium (NSM) (33), Penassay broth (Difco), TSS defined medium (8) with NH_4Cl as the nitrogen source and carbon sources as described, or Spizizen minimal medium (1) with 0.2% glucose or 1% Casamino Acids as the carbon source. All growth was at 37°C. Antibiotics (Sigma Chemical Co.) were used at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 5 μg/ml for selection and 0.1 μg/ml for induction; neomycin, 5 μg/ml; lincomycin, 25 μg/ml; and erythromycin, 1 μg/ml for selection and 0.1 μg/ml for induction. Amino acids were added at 50 μg/ml as required for auxotrophic strains.

Genetic techniques. *B. subtilis* chromosomal DNA isolation, transformation, and generalized transduction with phage

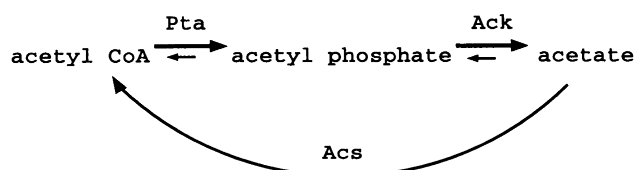

FIG. 1. Pathways for interconversion of acetate and acetyl-CoA. Acetate utilization in *B. subtilis* requires acetyl-CoA synthetase (Acs). Excretion of excess acetate occurs by a two-step pathway. Conversion of acetyl-CoA to acetyl phosphate utilizes phosphotransacetylase (Pta), and breakdown of acetyl phosphate to acetate (which results in synthesis of one ATP molecule) requires acetate kinase (Ack).

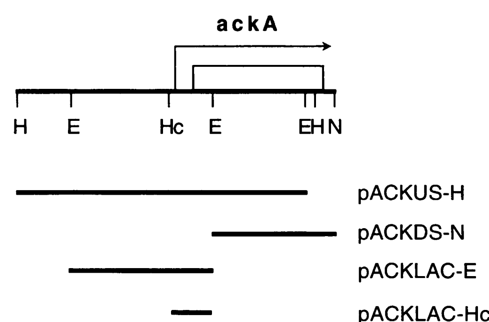

FIG. 2. Map of the *ackA* gene region. A region approximately 3 kb in length is shown. The box represents the AckA-coding sequence. The *ackA* transcript is shown by an arrow. Restriction sites: H, *Hin*dIII; E, *Eco*RI; Hc, *Hin*cII; N, *Nsi*I. Lines below the restriction map indicate the DNA segments present in the plasmids listed. pACKUS-H contains the 2.7-kb *Hin*dIII-*Eco*RI region, including most of *ackA* plus upstream DNA. pACKDS-N contains a 1.0-kb *Eco*RI-*Nsi*I region, starting within the AckA-coding region and extending just beyond the end of *ackA*. pACKLAC-E and pACKLAC-Hc include transcriptional fusions to a *lacZ* reporter gene and contain the 1.35-kb *Eco*RI and 0.34-kb *Hin*cII-*Eco*RI fragments, respectively.

PBS-1 were as previously described (15). Restriction endonucleases and DNA-modifying enzymes were purchased from New England BioLabs (Beverly, Mass.) and used as described by the manufacturer. Cloning in phage M13, preparation of single-stranded and double-stranded template DNAs, and dideoxynucleotide sequencing (Sequenase; United States Biochemicals) were carried out by using standard techniques. Synthetic oligonucleotides used as sequencing primers and hybridization probes were purchased from Genosys Biotechnologies, Inc. (Woodlands, Tex.). DNA sequence data were analyzed by using the PC/Gene system and the GenBank On-line Service (Intelligenetics, Inc.).

Hybridization techniques. The ACK oligonucleotide (5′-CATTGTTTGATGAAATGCTGTATCAAA-3′) was designed on the basis of the identification of a region of *E. coli ackA* conserved in a second *E. coli* gene which was also considered likely to encode an acetate kinase (see below). This region was chosen on the basis of high conservation in the two *E. coli* genes and low redundancy in codon selection. Codon selection for the ACK oligonucleotide was based on codon preferences found in *B. subtilis* highly expressed genes (11). Southern hybridization analysis of *B. subtilis* chromosomal DNA digests was carried out by standard techniques (32). Bacteriophage M13 libraries were screened by hybridization with 5′-^{32}P-labeled ACK oligonucleotide as described by Wei and Surzycki (40).

Primer extension analysis. RNA was isolated from strain BR151 as described by Wu et al. (42) from cells grown to 2 h after entry into stationary phase in NSM in the presence or absence of 1% (wt/vol) glucose. Oligonucleotide ACK4 (5′-CGCTCCTTTATACTCTG-3′), corresponding to positions 675 to 691 in Fig. 3, was 5′ end labeled and used in primer extension reactions with 20 μg of cellular RNA. The reverse transcription products were resolved on a denaturing 6% polyacrylamide gel, using as size standards DNA sequencing reaction products generated with the ACK4 oligonucleotide as primer.

Plasmid constructions. A map of the *ackA* region and plasmid constructs is shown in Fig. 2. Integrational plasmids were constructed by using derivatives of pGEM plasmids (Promega, Madison, Wis.) with the 0.85-kb *Eco*RI fragment and a drug resistance gene selectable in *B. subtilis* (*cat* and *neo* in pACKKO-CAT and pACKKO-NEO, conferring resistance to chloramphenicol and neomycin, respectively). The chromo-

TABLE 1. Bacterial strains

Strain	Genotype	Source and/or reference
B. subtilis		
BR151MA	*lys-3 trpC2*	10
WLN29	*aroGH trpC2 ccpA*::Tn*917lac*	16
BR151MAccp	*lys-3 trpC2 ccpA*::Tn*917lac*	MLS^r transformant of BR151MA, using WLN29 DNA
SMY	*Prototroph*	A. L. Sonenshein
ACKKO-CAT	*ackA*::pACKKO-CAT	Cm^r transformant of SMY, using pACKKO-CAT DNA
ACKKO-NEO	*lys-3 trpC2 ackA*::pACKKO-NEO	Neo^r transformant of BR151MA, using pACKKO-NEO DNA
SMYpDEBM13	*amyE*::pDEBM13	Cm^r Amy$^-$ transformant of SMY, using pDEBM13 DNA (10)
KS115	*cysA14 hisA1 leuA8 metC3 trpC2*	K. Sandman
1A119c	*pheA2 leuA164 argGH2 trpC2*	*Bacillus* Genetic Stock Center
1A92	*argGH2 aroG932 bioB141 sacA321*	*Bacillus* Genetic Stock Center
E. coli		
JM103	*endA1 supE44 sbcBC thi-1 rpsL* Δ(*lac-pro*)/F′ *traD36 lacI*^{q}*Z*Δ*M15 proAB*	24
DH5α	φ80d*lacZ*Δ*M15 endA1 recA1 hsdR17* (r_K^- m_K^+) *thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)U169	Bethesda Research Laboratories

somal regions flanking the 0.85-kb *Eco*RI fragment were isolated by excision of the integrated plasmids plus adjacent DNA by restriction endonuclease digestion of chromosomal DNA isolated from strains containing the integrated plasmids, ligation, and propagation in *E. coli*. Plasmid pACKUS-H contains the region upstream of the 0.85-kb *Eco*RI fragment, to a *Hin*dIII site 1.85 kb upstream; plasmid pACKDS-N contains the downstream region, to an *Nsi*I site located just past the end of the *ackA* coding region. Fusions to *lacZ* were constructed by using plasmid pFG328, a derivative of pBR322 containing a polylinker region upstream of a promoterless *lacZ* gene, and a *cat* gene selectable in *B. subtilis*. Plasmid pACKLAC-E contains the 1.35-kb *Eco*RI fragment, which includes the *ackA* promoter and amino-terminal coding region, as well as approximately 1 kb of upstream DNA; plasmid pACKLAC-Hc contains the 340-bp *Hin*cII-*Eco*RI fragment.

Acetate kinase assays. Cells were grown in Spizizen minimal medium containing 1.0% Casamino Acids as the carbon source until early logarithmic growth phase. Glucose was then added to 0.5% (wt/vol), and growth was continued for 90 min. Cells were harvested by centrifugation, resuspended in 50 mM KPO_4 (pH 7.2)–5 mM glutathione, and disrupted by sonication. Debris was removed by centrifugation at 15,000 × *g* for 15 min, and extracts were assayed at 30°C in the direction of acetyl phosphate formation by using hydroxylamine as described by Rose et al. (31). Lithium acetyl phosphate (Sigma Chemical Co.) was used as a standard. Protein concentrations in cell extracts were determined by the Bradford method (2), using a kit from BioRad, with bovine serum albumin as the standard.

β-Galactosidase measurements. Expression of *ackA* was monitored with *ackA-lacZ* transcriptional fusions constructed by using plasmid pFG328; the fusions were introduced into specialized transducing phage SPβ by homologous recombination so that they could be transferred in single copy into different host cell genomes. Cultures were maintained in early exponential growth for several generations by serial dilution in the appropriate growth medium so that adaptation to the growth medium was complete before the start of the experiment. β-Galactosidase assays were carried out as described by Miller (25), using toluene permeabilization of the cells.

Nucleotide sequence accession number. The sequence information reported in this study has been deposited in the GenBank data base under accession number L17320.

RESULTS

Cloning of the *B. subtilis ackA* gene. The sequence for the *E. coli ackA* gene has been reported (21). In the hope of identifying additional acetate kinase gene sequences, with the goal of selecting regions of the protein likely to be conserved in *B. subtilis*, we searched the GenBank data base for sequences with high similarity to *E. coli* AckA. This search revealed a single entry, corresponding to an unidentified open reading frame (ORF X) located downstream of the *tdc* operon of *E. coli*, which contains the gene for the biodegradative threonine dehydratase, involved in utilization of threonine under anaerobic conditions (35). Although ORF X was only 135 amino acids in length (9), inspection of downstream regions (34) showed that three shifts in the reading frame in regions of high G+C content would give a product 325 amino acids in length with extensive similarity with *E. coli* AckA. Although this putative (incomplete) gene has not been analyzed at the transcriptional level, its presence adjacent to or in an operon expressed during anaerobic growth suggests the possibility that this gene might also be expressed then.

The alignment of *E. coli* AckA with ORF X resulted in identification of a region at amino acids 149 to 157 of *E. coli* AckA which was highly conserved and contained a number of amino acids with low ambiguity in codon selection. Oligonucleotide ACK, complementary to this region and with *B. subtilis* codon preferences, was used as a hybridization probe with *B. subtilis* chromosomal digests. Single 0.85- and 2.8-kb bands were identified in *Eco*RI and *Hin*dIII digests, respectively (data not shown). The 0.8- to 0.9-kb size class of *Eco*RI-digested DNA was inserted into a bacteriophage M13 vector, and plaques which hybridized with the ACK oligonucleotide were identified. DNA sequence analysis revealed a 25-of-27 match to the ACK oligonucleotide probe. The cloned DNA contained an open reading frame which exhibited high similarity to both *E. coli* Ack gene products and corresponded to an internal fragment of the coding region. The flanking regions were isolated by integration of a plasmid containing the 0.85-kb *Eco*RI fragment into the *B. subtilis ackA* chromosomal locus by homologous recombination and excision of the plasmid plus adjacent chromosomal DNA. Plasmid pACKUS-H, obtained by digestion with *Hin*dIII, contains the upstream region (and 1 kb more than is shown in Fig. 3), and plasmid pACKDS-N contains the region downstream to an *Nsi*I site just past the end of *ackA*.

Sequence analysis of the *B. subtilis ackA* gene. The nucleotide sequence of a 1.9-kb region including *ackA* is shown in Fig. 3. The AckA open reading frame is 395 amino acids in length and is predicted to encode a protein with a molecular weight of 43,137; the *E. coli* AckA protein is 400 amino acids in length and has a molecular weight of 43,297 (22), while the sequence for ORF X is incomplete. The *B. subtilis* AckA-coding region is preceded by a sequence with strong similarity to *B. subtilis* translation initiation signals. A sequence with good adherence to the consensus for *B. subtilis* vegetative gene promoters recognized by $E\sigma^A$ was found further upstream, with a predicted transcription start point at position 606 in Fig. 3, 92 bp upstream from the probable AUG translation initiation codon. An additional open reading frame, for which no homolog was found in a search of the GenBank data base, was identified in the region upstream from *ackA*; an inverted repeat sequence, which could represent a factor-independent transcription termination site, was found in the region between the end of the open reading frame and the predicted *ackA* promoter.

Alignment of the *B. subtilis* AckA product with *E. coli* AckA and ORF X is shown in Fig. 4. The sequence identity of *B. subtilis* AckA to *E. coli* AckA was 43%; with inclusion of substitution of similar amino acids, the similarity was 60%. Over the region of ORF X available, the two *E. coli* proteins exhibit 44% identity and 60% similarity.

Transcriptional analysis. The start point for *ackA* transcription was determined by primer extension analysis (Fig. 5). A major initiation site was identified at position 606 of Fig. 3, corresponding exactly to the position predicted from inspection of the DNA sequence. The steady-state levels of the transcript were much higher in RNA isolated from cells grown in the presence of glucose, suggesting that *ackA* expression is induced by excess carbohydrate, which is consistent with a role for this gene product in excretion of acetate. These same RNA preparations were used in analysis of transcription of the *acsA* and *acuABC* genes, expression of which is repressed by glucose (12), indicating that the differences in expression are authentic and are not due to variations in the concentration or quality of the RNA preparations.

A second transcription initiation site, 7 bp upstream from the major site, was also identified. The levels of this transcript

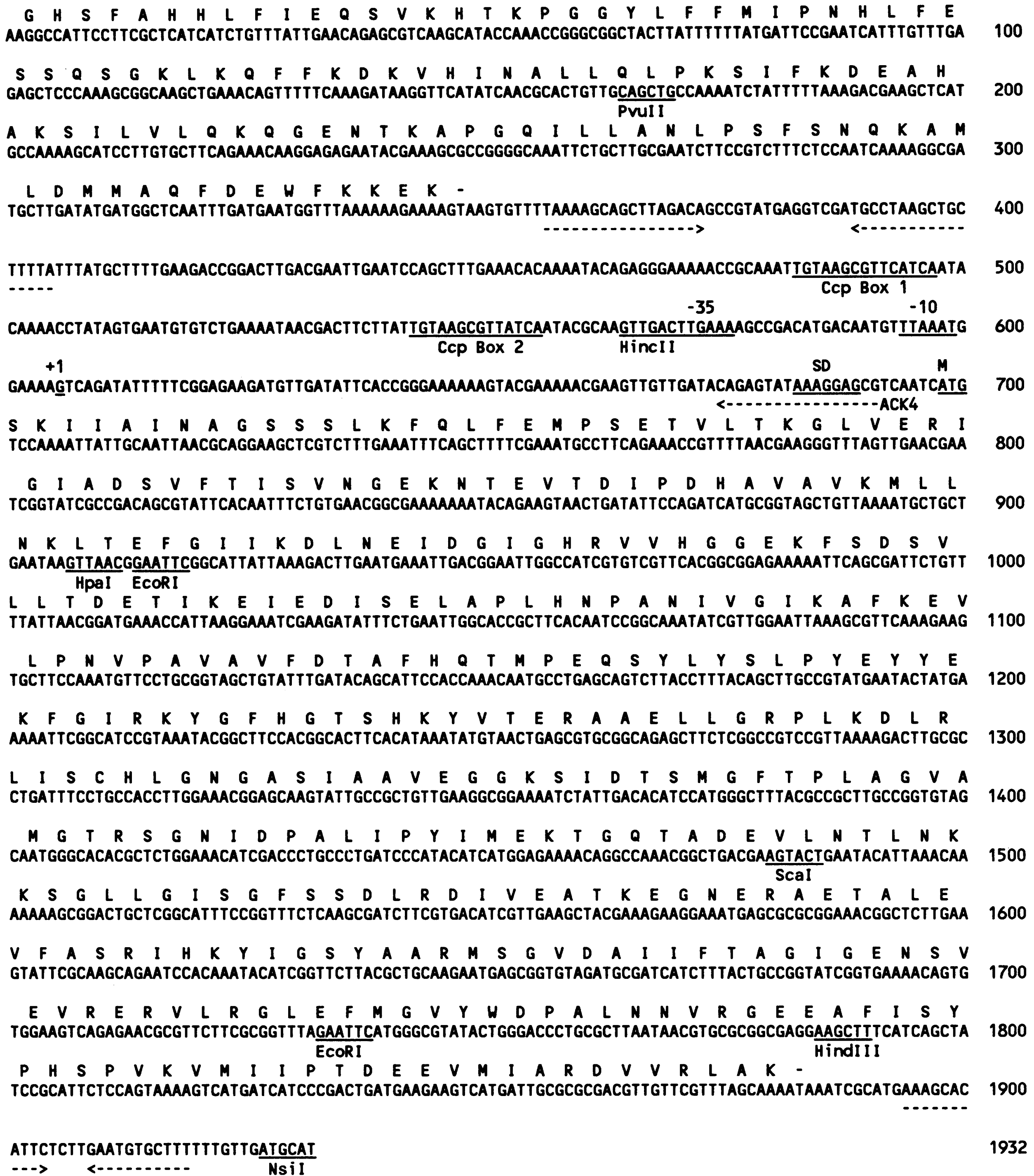

FIG. 3. DNA sequence of the *ackA* gene. The region shown includes a portion of an unidentified open reading frame located upstream of *ackA*. The predicted translation products are shown above the DNA sequence. Restriction endonuclease cleavage sites are underlined and labeled below the DNA sequence. The *ackA* promoter sequence (−35, −10, +1) and ribosome binding site (SD) are underlined and labeled above the DNA sequence. Putative transcriptional termination sites preceding and following the AckA-coding region are indicated by inverted dashed arrows. The position corresponding to the ACK4 oligonucleotide used for primer extension is labeled and indicated by a dashed arrow. Two sequences which closely resemble the *amyO* operator site of the *amyE* gene (27) are underlined and labeled (Ccp Box 1 and Ccp Box 2).

were very low and were not affected by growth in the presence of glucose. There is no obvious promoter sequence corresponding to a start site at this position. This band may represent a low-level constitutive transcript which contributes to basal-level *ackA* expression. A similar situation is found in the *E. coli fabA* gene, expression of which is activated by FadR protein during growth in the absence of exogenous fatty acids; a weak secondary transcript which initiates upstream from the

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BSUACK: -MS--KIIAINAGSSSLKFQLFEMPSETVLTKGLVERIGIADSVFTISVN   47
ECOACK: -MSSKLVLVLNCGSSSLKFAIIDAVNGEEYLSGLAECFHLPEARIKWKMD   49
ORF X : MNEFPVVLVINCGSSSIKFSVLDASDCEVLMSGIADGINSENAFL--SVN   48

BSUACK: GEKNTEVTDIPD-HAVAVKMLLNKLTEFGIIKDLNEIDGIGHRVVHGGEK   96
ECOACK: GNKQEAALGAGAAHSEALNFIVNTILAQKPELSAQ-LTAIGHRIVHGGEK   98
ORF X : GGEPAPL----AHHSYEGALKAIAFELEKRNLNDS-VALIGHRIAHGGSI   93

BSUACK: FSDSVLLTDETIKEIEDISELAPLHNPANIVGIKAFKEVLPNVPA--VAV  144
ECOACK: YTSSVVIDESVIQGIKDAASFAPLHNPAHLIGIEEALKSFPQLKDKNVAG  148
ORF X : FTESAIITDEVIDNIRRVSPLAPLHNYPNLSGIESAQQLFPGVTQ--VAL  141

BSUACK: FDTAFHQTMPEQSYLYSLPYEYYEKFGIRKYGFHGTSHKYVTERAAELLG  194
ECOACK: FDTAFHQTMPEESYLYALPYNLYKEHGIRRYGAHGTSHFYVTQEAAKMLN  198
ORF X : FDTSFHQTMAPEAYLYGLPWKYYEELGVRRYGFHGTSHRYVSQRAHSLLN  191

BSUACK: RPLKDLRLISCHLGNGASIAAVEGGKSIDTSMGFTPLAGVAMGTRSGNID  244
ECOACK: KPVEELNIITCHLGNGGSVSAIRNGKCVDTSMGLTPLEGLVMGTRSGDID  248
ORF X : LAEDDSALVVAHLGNGASICAVRNGQSVDTSMGMTPLEGLMMGTRSGDVD  241

BSUACK: PALIPYIMEKTGQTADEVLNTLNKKSGLLGISGFSSDLRDIVE---ATKE  291
ECOACK: PAIIFHLHDTLGMSVDAINKLLTKESGLLGLTEVTSDCRYVED-NYATKE  297
ORF X : FGPMSWVRRQTNQSLGDLERVVNKESGLLGISGHSSDLRVLGKSLHEGHE  291

BSUACK: GNERAETALEVFASRIHKYIGSYAARMSG-VDAIIFTAGIGENSVEVRER  340
ECOACK: ---DAKRAMDVYCHRLAKYIGAYTALMDGRLDAVVFTGGIGENAAMVREL  344
ORF X : ---RAQLAIKTFVHRIARHIAGHAASLR-RLDGIIFTGGIGENS...    331

BSUACK: VLRGLEFMGVYWDPALNNVR--GEEAFISYPHSPVKVMIIPTDEEVMIAR  388
ECOACK: SLGKLGVLGFEVDHERNLAARFGKSGFINKEGTRPAV-VIPTNEELVIAQ  393

BSUACK: DVVRLAK                                             395
ECOACK: DASRLTA                                             400
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FIG. 4. Alignment of the *B. subtilis* AckA (BSUACK), *E. coli* AckA (ECOACK), and ORF X protein sequences. Vertical lines indicate identical residues. Colons indicate conservative substitutions (A, S, and T; D and E; F, W, and Y; N and Q; R and K; and I, L, M, and V). Dashes indicate spaces inserted to maximize alignment.

normal FadR-regulated start site and does not respond to FadR was identified (17).

Insertional inactivation of *ackA*. The biological function of *ackA* in *B. subtilis* was explored by the generation of a mutant in which the gene was disrupted by integration of a plasmid within the coding sequence. The resulting strain, designated ACKKO-CAT, exhibited poor growth in any type of rich growth medium (tryptose blood agar base, LB medium, Penassay broth, or NSM). No effect on growth on TSS minimal medium with acetate as the carbon source was detected (11), indicating that this gene product is not required for acetate utilization. Growth of the *ackA* mutant in TSS defined medium with 1% Casamino Acids as the carbon source (Fig. 6) was identical to that of a wild-type control strain (which contains the same plasmid vector integrated at the *amyE* locus). Addition of 1.0% glucose increased both the growth rate and the growth yield of the wild-type strain but greatly inhibited growth of the ACKKO mutant. For the control strain, the doubling time decreased from 55 to 30 min in the presence of glucose, while for the mutant, the doubling time increased from 55 to 90 min. The final growth yield of the ACKKO strain in the presence of glucose was identical to that of the control strain, indicating that glucose could be utilized in the *ackA* mutant. *E. coli ackA* mutants also exhibit poor growth in rich media (39).

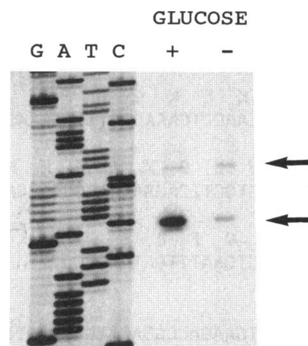

FIG. 5. Primer extension analysis of *ackA* transcription. The ACK4 oligonucleotide was end labeled and used in reverse transcription reactions with RNA isolated from *B. subtilis* cells grown to 2 h after entry into stationary phase in NSM in the absence (−) or presence (+) of 1% glucose. A sequencing ladder (GATC) generated by using the same oligonucleotide as primer was used as a size standard. Arrows indicate the positions of the primer extension products.

Acetate kinase enzyme activity. Isogenic wild-type and AckA$^-$ strains were grown in minimal medium with Casamino Acids as the carbon source, and then glucose was added for induction (see below). Acetate kinase activity, measured as formation of acetyl phosphate from acetate plus ATP, was 0.46 μmol/min/mg of protein in extracts of the wild-type strain and was dependent upon both acetate and ATP. No activity

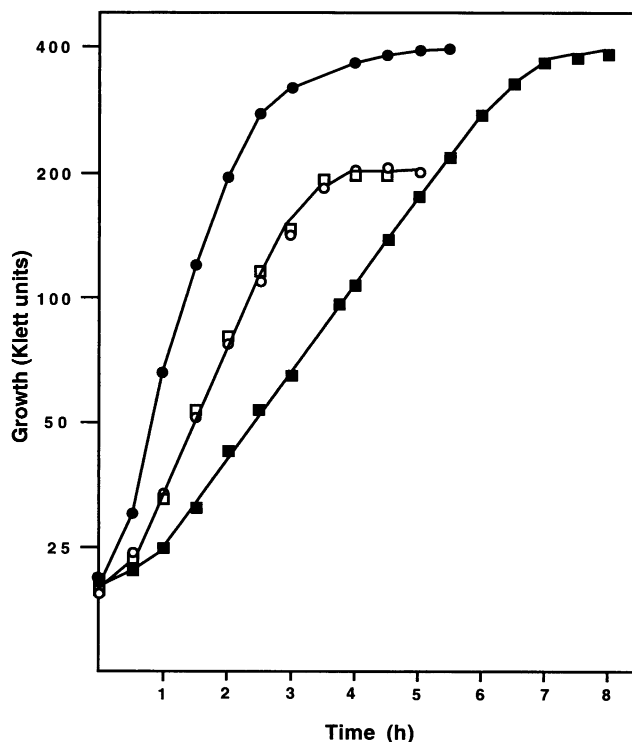

FIG. 6. Effect of glucose on growth of an AckA mutant. Cells were grown in TSS minimal medium with Casamino Acids (1%) as the carbon source (open symbols) or in TSS medium containing Casamino Acids and 1% glucose (filled symbols). Circles, wild-type strain (SMYpDEBM13); squares, AckA mutant (ACKKO-CAT).

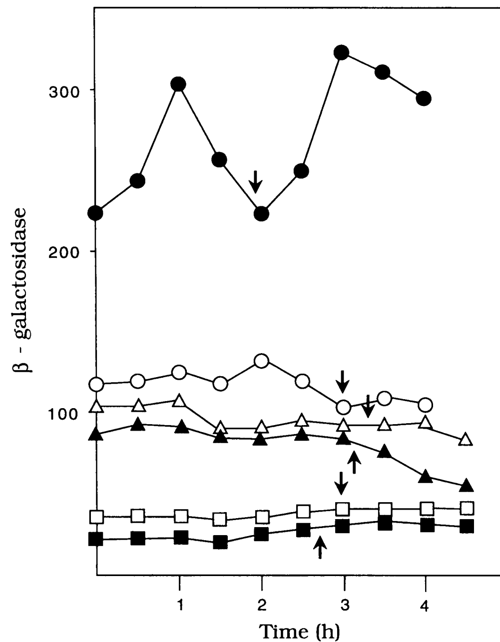

FIG. 7. Expression of *ackA-lacZ* transcriptional fusions in *B. subtilis*. Fusions were in single copy in an SPβ prophage. β-Galactosidase activity is expressed in Miller units (25). Cells were grown in TSS minimal medium containing 1% Casamino Acids as the carbon source (open symbols) or in TSS medium with 1% Casamino Acids and 1% glucose (filled symbols). Host strains were BR151MA (wild type; circles and squares) or BR151MAccp (CcpA$^-$; triangles). Fusions were derived from pACKLAC-E (circles and triangles) or pACK-LAC-Hc (squares). Vertical arrows indicate the time at which each culture entered into stationary phase.

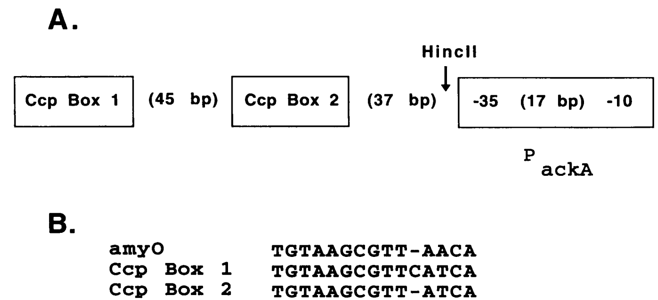

FIG. 8. *ackA* promoter region. (A) Diagram of the *ackA* promoter region. Boxed regions are the two sequences which resemble the *amyO* operator site (Ccp Box 1 and Ccp Box 2) and the *ackA* promoter sequence. The position of the *Hin*cII site used to construct the pACKLAC-Hc fusion, lacking the region upstream of the promoter, is shown. (B) Sequence comparison of *amyO* and Ccp boxes 1 and 2.

(<0.005 μmol/min/mg of protein) was detected in the *ackA*::pACKKO-NEO mutant.

Genetic mapping of the *ackA* gene. The position of the *ackA* gene on the *B. subtilis* chromosome was determined by phage PBS-1 generalized transduction, using the *ackA*::pACKKO-CAT mutant as the donor strain and strain KS115 as the recipient. Rescue of each of the auxotrophic markers of the recipient strain was selected, and transductants were screened for chloramphenicol resistance. Linkage of *cat* to *leuA* was 20%, while no linkage to *cysA*, *hisA*, *trpC*, or *metC* was detected. Three-factor transformation mapping was employed, using recipient strains with additional markers in the *leuA* region (data not shown). The predicted gene order for this region was *pheA leuA argGH ackA aroG*, indicating that *ackA* is located in the same region of the chromosome as *ccpA* and *rpsD*, which are located between *aroG* and *argGH* at 263° on the genetic map (15, 26). Overall two-factor genetic linkages by transformation were 6% to *pheA*, 7% to *leuA*, 50% to *argGH*, and 30% to *aroG*.

Expression of *ackA-lacZ* fusions. The nutritional conditions for *ackA* gene expression were analyzed by using transcriptional fusions to *lacZ* carried on an SPβ prophage. A fusion containing the entire *ackA* upstream region in a wild-type host strain exhibited low expression when grown in TSS minimal medium with 1% Casamino Acids as the carbon source, with little variation during the growth cycle (Fig. 7). Addition of 1.0% glucose resulted in expression that increased during exponential growth, peaked at mid-log phase, dropped transiently at the time of entry into stationary phase, and then increased during stationary phase; this complex pattern was reproducible. These results indicate that *ackA* expression is induced by growth in excess carbohydrate, consistent with the observed increase in the steady-state level of the transcript in glucose-grown cells. The basis for the fluctuations in expression during the growth cycle is unknown.

Introduction of the fusion into a CcpA$^-$ strain resulted in loss of stimulation of *ackA-lacZ* expression by glucose (Fig. 7). The CcpA$^-$ strain is clearly able to utilize the added glucose, since both the growth rate and growth yield of the mutant were increased by glucose addition (11), as observed for the wild-type strain. It therefore appears that induction of *ackA* transcription in response to excess glucose requires CcpA.

Identification of possible *cis*-acting sites for regulation by CcpA. Target sites for glucose repression by CcpA have been identified in the *amyE*, *acsA*, and *acuABC* genes, and mutation of these sites results in loss of repression by glucose (11, 27, 41). Since *ackA* expression was found to respond to CcpA, albeit in a pattern opposite to that found in the other target genes, the promoter region of *ackA* was searched for sites resembling *amyO*. Two such sites were found, centered 22 and 82 bp upstream of the −35 region of the *ackA* promoter region; the two sites, which are highly similar to *amyO*, differ from each other by a single nucleotide and are separated by 60 bp, center to center (Fig. 8); these sites would therefore be predicted to be located on the same face of the DNA helix. The functional role of this region was tested by construction of an *ackA-lacZ* fusion containing the *ackA* promoter region but lacking sequences upstream from the *Hin*cII site located immediately upstream of the −35 promoter sequence. This fusion exhibited low expression throughout growth and stationary phase and was not inducible by glucose (Fig. 7). These results indicate that sequences upstream from the *ackA* promoter are necessary for stimulation of *ackA* expression by glucose. The pattern of expression is similar to that observed in the CcpA$^-$ strain, suggesting that the target region for CcpA action is within the region deleted from the *Hin*cII fusion; the *amyO*-like sequences in this region are the most likely candidate sites for CcpA action. Expression of the *Hin*cII fusion in a CcpA$^-$ strain was identical to that in a wild-type strain (data not shown), indicating that CcpA exerts its effect through the DNA region deleted in this construct.

Expression of the *Hin*cII fusion was approximately twofold lower than expression of the intact fusion in a CcpA$^-$ host strain. The CcpA$^-$ mutant used in this study contains an insertion of Tn*917lac* in *ccpA*, but the level of β-galactosidase in this strain is very low, approximately 5 U throughout the

exponential and stationary phases, with no effect from the addition of glucose (11). It is therefore unlikely that the difference is due to *ccpA-lacZ* expression. Deletion of sequences upstream from the *ackA* promoter may result in reduced activity because of removal of sequences which enhance promoter activity, independent of CcpA; many *B. subtilis* promoters, including *ackA*, contain an A+T-rich region upstream of the promoter, removal of which may reduce promoter activity. Alternatively, sequences required for synthesis of the low-level constitutive transcript detected in the primer extension analysis may have been removed in the *Hin*cII fusion, resulting in reduced basal expression.

DISCUSSION

Little information is available about the control of carbon excretion in *Bacillus* spp. In this study, we have identified the *B. subtilis ackA* gene, which encodes acetate kinase. This enzyme catalyzes interconversion of acetate and acetyl phosphate and appears to operate primarily in the direction of acetate formation. Expression of *ackA* was shown to be induced by growth in the presence of excess glucose, and this induction was dependent on the CcpA gene product.

The *ackA* gene is located in the same region of the *B. subtilis* chromosome as *ccpA*, *acsA*, and *acuABC*. The *acsA* and *acuABC* genes are involved in utilization of acetate and acetoin, respectively, and their expression is repressed by CcpA in the presence of readily metabolizable carbon sources (11, 12). Localization of *ackA* to this same region was surprising, since *acsA* and *acuABC* are involved in utilization of secondary carbon sources, while *ackA* is involved in acetate excretion. It therefore appears that this region includes a cluster of genes involved in acetate production and utilization and that CcpA regulates the expression of all of these genes in response to glucose availability.

Similar *cis*-acting target sequences for glucose repression have been identified in the *amyE*, *acsA*, and *acuABC* genes; all of these sequences resemble classical operator sites like those found in the *E. coli lac* and *gal* operons. The CcpA protein is similar in structure to the *E. coli* LacI and GalR repressors (16). Direct interaction between CcpA and the *amyO* operator sequence has recently been demonstrated by in vitro DNase footprinting experiments (4), and it is likely that CcpA interacts similarly with the operator sites in *acsA* and *acuABC*. In all of these cases, the putative CcpA binding site is located either within the promoter sequence or downstream of the transcription start site. The *ackA* gene, expression of which is induced by CcpA in the presence of glucose, contains two sequences upstream of the promoter which closely resemble *amyO* and are therefore candidate sites for binding of CcpA. Removal of the region containing these sites resulted in the loss of glucose induction of *ackA* expression, indicating that this region is required for activation.

The similarity of the target sequences suggests that CcpA can bind to all of these sites; the difference in the effect of CcpA binding (induction for *ackA* versus repression for *amyE*, *acsA*, and *acuABC*) may be dependent on the location of the binding site relative to the promoter sequence, so that binding of CcpA upstream results in activation, while binding within the promoter or downstream results in repression. Further studies will be needed to demonstrate a direct interaction between CcpA and these target sites and to demonstrate the effect of location relative to the promoter sequence. There are numerous examples of regulatory proteins which can act as both repressors and activators of gene expression; the variation in function depends on the presence of effector molecules or binding to different target sites. In general, negative effects are correlated with target sites located within or downstream of the promoter sequence, while positive effects are associated with target sites located upstream of the promoter (5). Our results suggest that binding of CcpA to both repressing and activating sites is similarly stimulated by growth in excess carbohydrate.

The CcpA protein plays a central role in regulation of the interconversion of acetate and acetyl-CoA, since it acts as a repressor for *acsA* and as an activator for *ackA*. Mutations in *ccpA* are therefore likely to affect the pools of acetyl-CoA, acetyl phosphate, and acetate. Phosphorylation of the regulatory proteins in several two-component signal transduction pathways in *E. coli*, including chemotaxis (19), the Pho regulon (39), and the Ntr regulon (7), as well as in the *B. subtilis* competence system (30), has been shown to occur in vitro with acetyl phosphate as the phosphate donor, bypassing the need for autophosphorylation of the sensor protein. Perturbations in acetate metabolism have been shown to affect chemotaxis (6, 19) and the expression of the Pho and Ntr regulons (7, 39) in vivo in *E. coli*, suggesting that this effect may have physiological relevance (23, 38). The availability of *ackA* and the ability to control its expression will provide valuable information about the roles of acetyl-CoA and acetyl phosphate in cellular physiology and regulation of gene expression in *B. subtilis.*

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