JOURNAL OF BACTERIOLOGY, Apr. 2001, p. 2497–2504 Vol. 183, No. 8
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.8.2497–2504.2001

Regulation of the Acetoin Catabolic Pathway Is Controlled by Sigma L in *Bacillus subtilis*

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Received 22 September 2000/Accepted 23 January 2001

Bacillus subtilis **grown in media containing amino acids or glucose secretes acetate, pyruvate, and large quantities of acetoin into the growth medium. Acetoin can be reused by the bacteria during stationary phase when other carbon sources have been depleted. The *acoABCL* operon encodes the E1α, E1β, E2, and E3 subunits of the acetoin dehydrogenase complex in *B. subtilis*. Expression of this operon is induced by acetoin and repressed by glucose in the growth medium. The *acoR* gene is located downstream from the *acoABCL* operon and encodes a positive regulator which stimulates the transcription of the operon. The product of *acoR* has similarities to transcriptional activators of sigma 54-dependent promoters. The four genes of the operon are transcribed from a −12, −24 promoter, and transcription is abolished in *acoR* and *sigL* mutants. Deletion analysis showed that DNA sequences more than 85 bp upstream from the transcriptional start site are necessary for full induction of the operon. These upstream activating sequences are probably the targets of AcoR. Analysis of an *acoR′-′lacZ* strain of *B. subtilis* showed that the expression of *acoR* is not induced by acetoin and is repressed by the presence of glucose in the growth medium. Transcription of *acoR* is also negatively controlled by CcpA, a global regulator of carbon catabolite repression. A specific interaction of CcpA in the upstream region of *acoR* was demonstrated by DNase I footprinting experiments, suggesting that repression of transcription of *acoR* is mediated by the binding of CcpA to the promoter region of *acoR*.**

During the growth of cultures of *Bacillus subtilis*, several products have been identified in the growth medium, such as lactate, acetate, succinate, acetoin, butanediol, and ethanol (5).

Acetoin (3-hydroxy 2-butanone) is a major catabolic product of *B. subtilis* grown aerobically in glucose media. Since it is neutral, this metabolite allows the bacteria to degrade large amounts of glucose without substantial acidification of the growth medium. Acetoin also serves as a carbon storage compound which is secreted into the growth medium and later reimported. In *B. subtilis*, the products of two genes, *ilvBN* (acetohydroxy acid synthase) and *alsS* (α-acetolactate synthase), are involved in the production of acetolactate from pyruvate. Acetolactate is converted to acetoin by spontaneous decarboxylation at low pH or via the action of *alsD*, encoding an acetolactate decarboxylase (37). Acetoin is reutilized during stationary phase when other carbon sources have been depleted. Many bacterial species are able to degrade acetoin: *Micrococcus urea* (20), *Alcaligenes eutrophus* (12), *Enterococcus faecalis* (10), *Pelobacter carbinolicus* (34), *Klebsiella pneumoniae* (9), and *Clostridium magnum* (22). Three genes forming an operon, *acuABC*, have been described in *B. subtilis*. The roles of the corresponding gene products are still unknown (16). Inactivation of the first gene of this operon resulted in diminished growth on acetoin and butanediol. Since utilization of acetoin is reduced but not abolished in an *acuABC* mutant, this result suggested that there is more than one pathway for acetoin utilization in *B. subtilis*. Recently, another gene cluster, the *aco* operon encoding the multicomponent acetoin dehydrogenase enzyme complex, was sequenced (18, 23). A plasmid encoding part of the α subunit of the acetoin dehydrogenase E1 was used to disrupt *acoA*, the first gene of this operon. This mutant was impaired in the expression of acetoin dehydrogenase E1 activity and for depletion of acetoin from the growth medium, indicating that this operon is the main system involved in the catabolism of acetoin (18). However, very little was known about the regulation of transcription of the *aco* operon in *B. subtilis*. The product of the *acoR* gene, which is located downstream from the *aco* operon, has similarities to transcriptional activators of sigma 54-dependent promoters. A putative −12, −24 promoter is located upstream from the *acoA* gene, strongly suggesting that the SigL sigma factor is necessary for its transcription. We studied the regulation of the expression of the *aco* operon in *B. subtilis* and found that transcription was strongly induced in the presence of acetoin in the growth medium and depended upon the presence of both AcoR and SigL.

MATERIALS AND METHODS

Bacterial strains and culture media. The *B. subtilis* strains used in this work are listed in Table 1. *Escherichia coli* TGI [K-12 Δ(*lac pro*) *supE thi hsd5*/F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15] was used for cloning experiments. *E. coli* was grown in Luria-Bertani broth (38), and *B. subtilis* was grown in SP medium (8 g/liter of nutrient broth [Difco], 1 mM $MgSO_4$, 10 mM KCl, 0.5 mM $CaCl_2$, 10 μM $MnCl_2$, 2 μM $FeSO_4$) or in CSK medium. CSK medium is C medium (28) supplemented with potassium succinate (6 g/liter) and potassium glutamate (8 g/liter).

Transformation and phenotype characterization. Standard procedures were used to transform *E. coli* (38), and transformants were selected on Luria-Bertani broth plates containing ampicillin (100 μg/ml). *B. subtilis* was transformed with plasmid or chromosomal DNA as previously described (1, 28), and transformants were selected on SP medium plates containing chloramphenicol (5 μg/ml), kanamycin (5 μg/ml), erythromycin (1 μg/ml) plus lincomycin (25 μg/ml), or spectinomycin (60 μg/ml). Amylase activity in *B. subtilis* was detected after growth on

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TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype or description	Source or reference
168	*trpC2*	1
QB5407	*trpC2 ccpA*::*spc*	36
QB5505	*trpC2 sigL*::*aphA3*	6
QB7700	*trpC2 amyE acoA′-′lacZ*	This work
QB7701	*trpC2 amyE acoA′-′lacZ ccpA*::*spc*	This work
QB7702	*trpC2 amyE acoA′-′lacZ sigL*::*aphA3*	This work
QB7704	*trpC2 amyE acoA′-′lacZ acoR*::*aphA3*	This work
QB7713	*trpC2 acoR*::pDIA5307	This work
QB7714	*trpC2 acoR*::pDIA5307 pACOR1	This work
QB7719	*trpC2 amyE acoA′-′lacZ*(ΔB)	This work
QB7720	*trpC2 amyE acoA′-′lacZ*(ΔC)	This work
QB7721	*trpC2 amyE acoA′-′lacZ*(ΔD)	This work
QB7724	*trpC2 acoA*::pMutin4	This work
QB7725	*trpC2 acoB*::pMutin4	This work
QB7726	*trpC2 acoC*::pMutin4	This work
QB7727	*trpC2 acoL*::pMutin4	This work
QB7728	*trpC2 amyE acoA′-′lacZ acuABC*::*aphA3*	This work
QB7733	*trpC2 acoR*::pDIA5307 *ccpA*::*spc* pACOR1	This work

tryptose blood agar base (Difco) containing 10 g of hydrolyzed starch per liter (Connaught). Starch degradation was detected by sublimating iodine onto the plates.

DNA manipulations. Standard procedures were used to extract plasmids from *E. coli* (38). Restriction enzymes, phage T4 DNA polymerase, phage T4 DNA ligase, and phage T4 polynucleotide kinase were used as recommended by the manufacturers. DNA fragments were purified from agarose gels with a Prep-A-Gene kit (Bio-Rad Laboratories). The PCR technique with *Thermus aquaticus* DNA polymerase was used for amplification. The oligonucleotide primers used included mismatches to create restriction sites.

Plasmid constructions. pAC5 (29), a derivative of pAF1 (11), carries the pC194 chloramphenicol resistance gene *cat* and a *lacZ* gene between two fragments of the *B. subtilis amyE* gene. PCR was used to introduce *Eco*RI restriction sites at various positions upstream from *acoA*. PCR was performed with one oligonucleotide (5′-GGAGGATCCTCAGTTAATGACAAGCCTTC-3′) corresponding to the coding sequence of the *acoA* gene (codons 7 to 13) and one oligonucleotide corresponding to various positions in the *acoA* promoter region. The *Eco*RI-*Bam*HI restriction fragments generated were inserted between the *Eco*RI and *Bam*HI restriction sites of pAC5, creating translational fusions between codon 13 of *acoA* and codon 8 of *lacZ*. The DNA sequences of the different PCR fragments were verified by direct sequencing of the various corresponding plasmids. The resulting plasmids were linearized at the single *Pst*I restriction site and integrated into the chromosome of strain 168 by homologous recombination at the *amyE* locus using chloramphenicol selection. The integrants carrying the translational fusions were named QB7700, QB7719, QB7720, and QB7721.

Gene disruptions and transcriptional fusions with pMutin4 (40) were constructed by PCR amplification of an internal segment of the target gene, ligation of the amplified DNA fragment into pMutin4, transformation of *E. coli*, and then insertion of the plasmid into the *B. subtilis* chromosome. The following oligonucleotides were used for PCR amplifications: 5′-GCCGAAGCTTGCCCAGGG AGTGCTTCCC-3′ and 5′-CGCGGATCCAGCAGCCGCCCGATCGGC-3′ for the *acoA* gene, 5′-GCCGAAGCTTGTCGCCGGGGGAGCGGCG-3′ and 5′-CGCGGATCCGACCTGCTTGCCGACTGC-3′ for the *acoB* gene, 5′-GCC GAAGCTTGGCGGTAAAAGTAGTGATG-3′ and 5′-CGCGGATCCGATC GTCAGCTGCGCGC-3′ for the *acoC* gene, and 5′-GCCGAAGCTTGACCGG CTGATCCCGGCT-3′ and 5′-CGCGGATCCGCCGATCTTCACATCCCC-3′ for the *acoL* gene. The target genes were interrupted by Campbell-type crossover integration. The integration of the recombinant plasmids fuses the target genes to the *lacZ* gene, leading to a transcriptional fusion.

The wild-type *acoR* gene was disrupted as follows. Two DNA fragments encoding the amino-terminal and the carboxy-terminal parts of *acoR* were synthesized by PCR with the following pairs of oligonucleotides, respectively: (i) 5′-GAAGAATTCGAAGGGGCATGCTGGACAGAAAC-3′ and 5′-GGAGG ATCCCTGCCAATCCGGCATTGAGGTTC-3′ and (ii) 5′-CTGCTGCAGCG CGATCGCACCGAGGATATCCC-3′ and 5′-AAGAAGCTTTCCAGCGGCT TCCAGTAAAGCGG-3′ . The two DNA fragments were ligated into pHT181 (25) on each side of a DNA fragment containing *aphA3* (39), leading to pACOR2. The interrupted gene was introduced into the chromosome of strain QB7700 to give strain QB7704. The wild-type *acoR* gene was cloned as follows. Strain QB7704 was transformed with a library of *B. subtilis* DNA established in *E. coli* by using the shuttle vector pHT315 (24). Transformants were isolated on CSK-erythromycin plates containing 10 mM acetoin and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Several blue colonies were isolated. Plasmid DNA was extracted from cultures of one of these transformants, and the insert was characterized by DNA sequencing. The corresponding plasmid, which contained the entire wild-type *acoR* gene, was called pACOR1.

An *acoR′-′lacZ* transcriptional fusion was constructed in pDIA5307 (4) by PCR amplification of an internal fragment of the *acoR* gene, ligation of the amplified DNA fragment into pDIA5307, transformation of *E. coli*, and insertion of the recombinant plasmid into the chromosome of the 168 strain of *B. subtilis*, leading to strain QB7713. PCR amplification was done with two oligonucleotides: 5′-GAAGAATTCGAAGGGGCATGCTGGACAGAAAC-3′ and 5′-GG AGGATCCCTGCCAATCCGGCATTGAGGTTC-3′ .

Reverse transcriptase mapping of the mRNA start point in the *acoA* gene. Total RNA was isolated from *B. subtilis* 168 grown in CSK medium with or without 10 mM acetoin as the inducer. Exponentially grown cells were harvested at an optical density at 600 nm of 0.5, and RNA was extracted (15). One oligonucleotide (5′-CCTCAGTTAATGACAAGCCTTCTCG-3′) complementary to the *acoA* coding sequence was labeled with 10 U of polynucleotide kinase and 0.37 MBq of [γ^{-32}P]ATP (15 TBq/mmol; Amersham). The DNA primer was elongated, and the product was analyzed as previously described (27).

DNase I footprinting. DNA fragments used for DNase I footprinting were prepared by PCR using *Pfu* polymerase (Stratagene) and 20 pmol of each primer (5′-GGCGCTGTTAAGCACGATCGGCCTTGCG-3′ and 5′-CCGTTTTCTT AATCGGGCTTCGTCAACC-3′), one of which was previously labeled with T4 polynucleotide kinase (New England Biolabs) and [γ^{32}P]ATP. The labeled PCR products were purified with the Qiagen PCR purification kit. Binding of CcpA, HPr, or HPr-Ser-P to these DNA probes was performed in a 20-μl volume containing 2×10^5 cpm of the ^{32}P-labeled DNA fragments and 1 μg of poly(dI-dC) in 100 mM KCl, 10 mM HEPES (pH 7.6), 0.1 mM EDTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 10% glycerol. The DNA binding reaction was performed in the presence of 2 μM CcpA and 10 μM either HPr or HPr-Ser-P by incubating the assay mixture for 45 min at room temperature. The concentrations of $MgCl_2$ and $CaCl_2$ were adjusted to 1 and 0.5 mM, respectively, and 20 ng of DNase I (Worthington Biochemical, Freehold, N.J.) was added. The mixture was incubated at room temperature for 1 min, and the reaction was stopped by the addition of 7 volumes of stop buffer (0.4 M sodium acetate, 50 μg of calf thymus DNA/ml, 2.5 mM EDTA) followed by phenol extraction. The DNA fragments were ethanol precipitated, and an equivalent number of cpm (2×10^5) from each reaction was loaded on 7% polyacrylamide sequencing gels. A+G Maxam and Gilbert reactions (32) were carried out on the appropriate ^{32}P-labeled DNA fragments, and the products were loaded alongside the DNase I footprinting reactions. Gels were dried and analyzed by autoradiography.

β-Galactosidase assays. *B. subtilis* cells containing *lacZ* fusions were grown to an optical density at 600 nm of 1. β-Galactosidase specific activities were deter-

TABLE 2. Expression of the *acoABCL* operon and *acoR*

Strain	Relevant genotype	β-Galactosidase specific activity (Miller units/mg of protein)		
		CSK	CSK + 10 mM acetoin	CSK + 10 mM acetoin + 0.5% glucose
QB7700	*acoA'-'lacZ*	145	48,975	655
QB7701	*acoA'-'lacZ ccpA::spc*	535	55,210	50,035
QB7704	*acoA'-'lacZ acoR::aphA3*	10	10	3
QB7702	*acoA'-'lacZ sigL::aphA3*	5	2	3
QB7713	*acoR'-'lacZ*	155	140	10
QB7714	*acoR'-'lacZ* pACOR1	125	145	5
QB7724	*acoA'-'lacZ* pMutin4	355	3,875	ND[a]
QB7725	*acoB'-'lacZ* pMutin4	470	4,230	ND
QB7726	*acoC'-'lacZ* pMutin4	95	4,740	ND
QB7727	*acoL'-'lacZ* pMutin4	45	3,765	ND
QB7733	*acoR'-'lacZ* pACOR1 *ccpA::spc*	215	230	220

[a] ND, not determined.

mined as previously described and are expressed as Miller units per milligram of protein (7). The values reported are averages of at least three independent assays.

Acetoin assays. The acetoin assays were carried out as previously described (16) except that the reactions were done at room temperature for 15 min.

Glucose assays. The glucose assays were performed by an enzymatic method using glucose oxidase and peroxidase as recommended by the manufacturer (Boehringer).

RESULTS

Induction of the *aco* operon in response to acetoin or glucose availability. Four genes which probably form an operon encode the E1α, E1β, E2, and E3 subunits of the acetoin dehydrogenase multienzyme complex (18). It was previously shown that utilization of acetoin strongly depends upon the culture conditions. Addition of acetoin led to an increase in the specific activity of acetoin dehydrogenase, whereas addition of acetoin with glucose prevents this increase. An *acoA'-'lacZ* translational fusion was constructed and used to elucidate the regulation of the expression of the *aco* operon. The expression of the hybrid gene was studied in strain QB7700 grown in minimal CSK medium with and without 10 mM acetoin as the inducer. *lacZ* expression was strongly induced by acetoin in the growth medium, as shown in Table 2. The *acuABC* gene cluster plays a role in acetoin catabolism. The product of *acuC* shares similarities with eukaryotic histone deacetylase (16). In eukaryotes, histone deacetylation plays an important role in transcriptional regulation of cell cycle progression and developmental events. We constructed a strain in which the *acuABC* operon was deleted. The mutation was introduced by transformation into QB7700, leading to strain QB7728. We did not observe any modification of the induction of the *acoA'-'lacZ* fusion, and the repression effect observed in the presence of glucose was similar to that observed in the parental strain QB7700 (not shown). This indicates that the *acu* operon has no effect on the expression of the *aco* operon in *B. subtilis*. Addition of glucose repressed the expression of the *lacZ* fusion. CcpA, a member of the LacI-GalR family of repressors, is a regulator of catabolite repression in *B. subtilis*. It is a negative regulator of carbon utilization genes and is a positive effector of genes involved in the biosynthesis and secretion of acetate and acetoin (17, 19). The role of CcpA in glucose control was investigated by testing the effect of a Δ*ccpA* mutation on the regulation of the *aco* operon. Chromosomal DNA from strain QB5407 *ccpA::spc* was introduced by transformation into strain QB7700, leading to strain QB7701. The effect of glucose was tested by comparing the β-galactosidase activity after the growth of strain QB7701 in CSK acetoin medium and in CSK acetoin glucose medium (Table 2). The expression of the *acoA'-'lacZ* fusion remained inducible by acetoin but was not repressed by glucose in the absence of CcpA in the cell.

It was previously shown that different carbon sources influence the ability of *B. subtilis* to degrade acetoin (26). To assess the relationships among acetoin production, glucose degradation, and induction of the *aco* operon, strain QB7700 was grown in CSK medium containing 42 mM glucose. Glucose, acetoin, and *lacZ* were assayed at various times for 24 h (Fig. 1). During the exponential phase of growth, the glucose concentration decreased rapidly while acetoin production increased to 35 mM, indicating that most of the glucose was converted to acetoin in the culture medium. As expected, the acetoin concentration began to decline when glucose was completely metabolized. In parallel, the *acoA'-'lacZ* fusion present in the QB7700 strain was induced, indicating that acetoin produced during aerobic growth in the presence of glucose induced the *aco* operon. Strikingly, the complete disappearance of glucose from the medium marked the start of transcription of the *aco* operon.

Induction of expression of *acoABCL* genes. The plasmid pMutin4 was used to construct transcriptional fusions with the *lacZ* gene. Integration of pMutin4 fuses the target genes to the *lacZ* gene and allows the expression of downstream genes from an IPTG-inducible promoter, p*spac*. These fusions were integrated by homologous recombination into the *acoABCL* genes in the chromosome, leading to strains QB7724, QB7725, QB7726, and QB7727, respectively. The recombination events were mediated by DNA fragments generated by PCR and corresponding to internal parts of each gene. These events inactivated the corresponding genes and fused them to the *lacZ* gene. The β-galactosidase activities of strains QB7724, QB7725, QB7726, and QB7727 were assayed after they were cultured in CSK medium containing or not containing acetoin (Table 2). The presence of 10 mM acetoin led to 10- to 100-fold-higher *lacZ* expression than in its absence. The induction by acetoin indicates that these four genes are coexpressed and

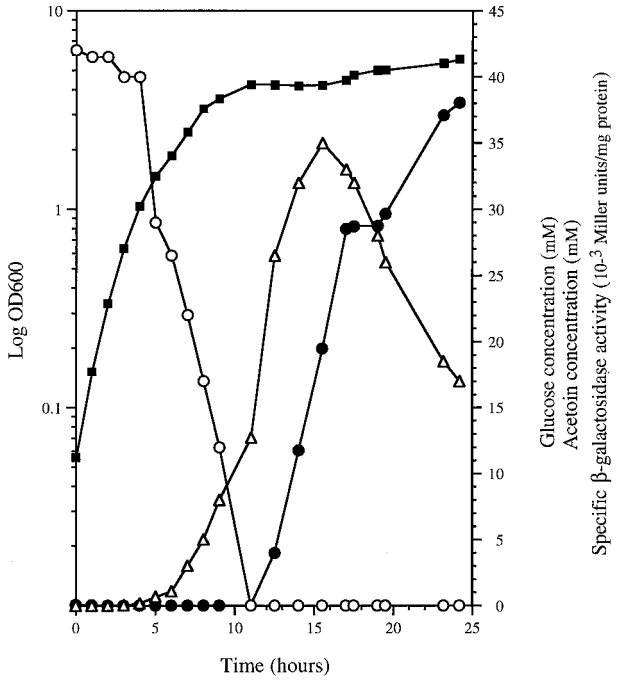


FIG. 1. Induction of the *acoA'-'lacZ* fusion after growth in glucose medium. *B. subtilis* strain QB7700 was cultured in CSK medium containing 42 mM glucose at 37°C. The optical density of the culture, at 600 nm (OD 600) was followed for 24 h, and the glucose and acetoin concentrations and LacZ activity were assayed. ■, optical density at 600 nm; ○, glucose concentration; Δ, acetoin concentration; ●, β-galactosidase specific activity.

probably belong to the same transcriptional unit. The level of β-galactosidase expression in strains containing transcriptional fusions constructed with pMutin4 was lower than that of the *acoA'-'lacZ* translational fusion in the vector pAC5. This is probably due to better translation of the *acoA'-'lacZ* mRNA. The *acoR* gene is located immediately downstream from the *aco* operon. Addition of 1 mM IPTG to the growth media of strains QB7724 and QB7727 did not change the *lacZ* expression (not shown). This result indicates that the expression of the *acoR* gene is not affected by the p*spac* promoter inserted into the *acoA* or *acoL* gene. This could be due to premature termination of transcription initiated at the p*spac* promoter, suggesting that the *aco* operon and the *acoR* genes are distinct transcriptional units.

The *acoR* and *sigL* gene products are involved in *aco* expression. The four genes, *acoABCL*, of the operon are followed by *acoR*, a gene which encodes a peptide of 67 kDa with similarities to activators of sigma 54-dependent promoters. This family of activators contains a region of 220 to 240 residues called the central domain, which is specifically required for the formation of open complexes between RNA polymerase and the −12, −24 promoters, probably by interacting with the RNA polymerase or with sigma 54. An *aphA3* cassette was inserted into the *acoR* gene by a double-crossover event. This mutation, which inactivates the *acoR* gene, was introduced into the chromosome of QB7700, leading to strain QB7704. β-Galactosidase activity was assayed in QB7704 cultures in CSK medium with or without acetoin and glucose (Table 2). The product of *acoR* was found to be necessary for the expression of the operon. This strongly suggests that the promoter of the *aco* operon is recognized by an RNA polymerase associated with the SigL sigma factor. Therefore, a *sigL*::*aphA3* null mutation was introduced by transformation into QB7700, leading to strain QB7702. The β-galactosidase activity was assayed in cultures of this strain grown in CSK medium with or without acetoin as the inducer (Table 2). There was no LacZ activity, indicating that a SigL-dependent promoter is involved in the transcription of the *aco* operon.

Promoter and control regions located upstream from the *acoABCL* operon. In gram-negative and gram-positive bacteria, all promoters recognized by holoenzyme containing σ^{54} possess at least one conserved GG doublet around position −26 followed by a GC doublet at a distance of 10 bp (2, 33). The transcription start site of *acoABCL* was mapped by primer extension using reverse transcriptase (Fig. 2). RNA was extracted from uninduced cells grown in CSK medium or induced cells grown in CSK medium containing 10 mM acetoin. A unique band was observed when RNA extracted under induced conditions was used, allowing the definition of the promoter. An alignment of the deduced promoter region with five other −12, −24 SigL-dependent promoters from *B. subtilis* is shown in Fig. 3. The promoter contains −12 and −24 regions identical to those observed in sigma 54-dependent promoters, for instance, the levanase and *rocABC* promoters (4, 6). A second DNA sequence with similarities to the −12, −24 promoter is present 135 bp upstream from the transcription start site. A DNA fragment lacking this similar sequence was synthesized by PCR and fused upstream from the *lacZ* gene. This construction was reintroduced at the *amyE* locus of *B. subtilis* by using the plasmid pDH32 (35). The resulting strain did not express LacZ activity in CSK medium containing acetoin as the inducer (not shown). This result indicates that only one −12, −24 promoter identified by primer extension (shown in Fig. 2) is involved in the transcription of the *acoABCL* operon.

The *aco* operon requires upstream sequences for its expression. Central-domain activators contact σ^{54}-holoenzyme through DNA looping after binding to the enhancer, also called upstream activating sequences (UAS), typically 80 to 60 bp upstream from the transcription start site. A notable exception is *rocG* in *B. subtilis*, which is transcribed by a SigL-containing RNA polymerase and requires RocR, a member of the NtrC/NifA family of proteins. Unlike other σ^{54}-dependent genes, *rocG* has no UAS; instead, its expression is dependent on a sequence called DAS (downstream activating sequence) located downstream from the *rocG* coding region. This DAS also serves as a UAS for *rocABC* (3). To identify any such sequences associated with *acoA* expression, deletions ending upstream from the transcriptional start site were introduced. A set of DNA fragments from which part of the upstream region was missing was obtained by PCR synthesis (see Materials and Methods). These fragments were then inserted upstream from the *lacZ* gene in pAC5. The deletion end points are indicated in Fig. 4. The fusions were reintroduced as single copies at the *amyE* locus of *B. subtilis*. The levels of *lacZ* expression in this strain were analyzed and are shown in Fig. 4. In the ΔB and ΔC deletions, *lacZ* expression was identical to that obtained in QB7700. In the ΔD deletion, *lacZ* expression was strongly reduced, indicating that full induction of the *aco* operon re-

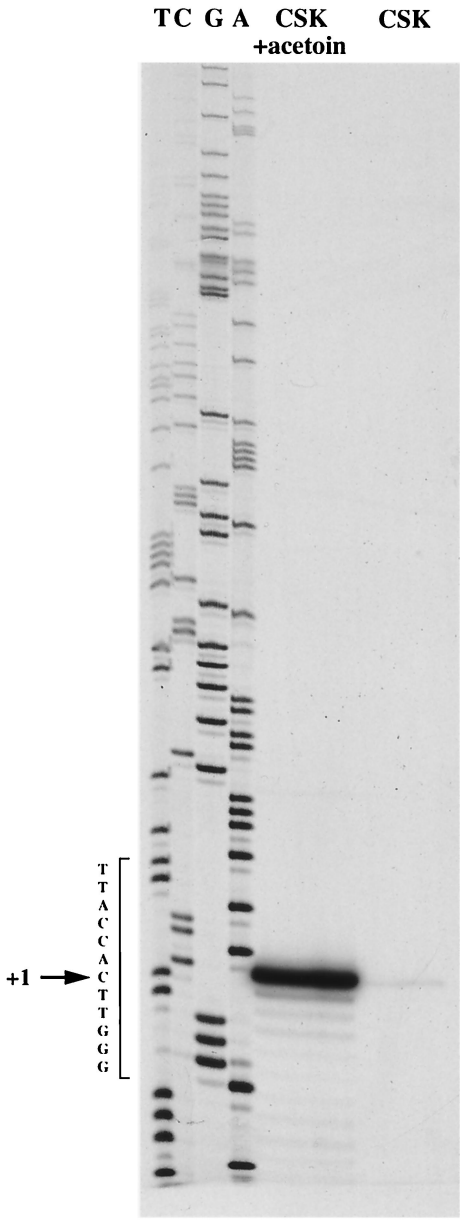


FIG. 2. Reverse transcriptase mapping of the transcriptional start site of the *acoA* gene. RNA was extracted from *B. subtilis* 168 grown in the presence (+ acetoin) or absence of 10 mM acetoin. The position of the cDNA-extended fragment was compared to that of fragments obtained by sequencing an M13 recombinant phage containing the promoter region, with the same oligonucleotide used as a primer. The transcriptional start site is indicated by an arrow.

quires DNA sequences located between positions −123 and −85. Interestingly, this DNA region includes three copies of a hexanucleotide sequence, 5′-GAGACA-3′ , and also a palindromic sequence of 11 bp centered on position −91. Possibly these direct repeats or the palindromic sequence are involved in the binding of AcoR.

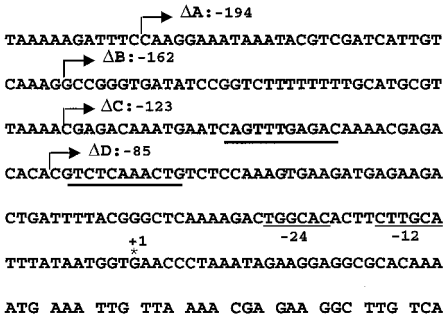
Analysis of *acoR* gene expression. The intergenic region located between the end of *acoL* and the beginning of *acoR* is 118 bp long, suggesting that *acoR* may be part of the *acoABCL*

		-24		-12	
p*acoA*	AAAAGAC	TGGCA	CACTT	CTTGCATT	TATAATGGTGAACCC
p*lev*	ACTGTGT	TGGCA	CGATC	CTTGCATT	ATATATGGATGTACA
p*rocABC*	AAGAAAA	TGGCA	TGATT	CTTGCATT	TTTATTCATATGCGA
p*rocG*	CAAAAGC	TGGTA	CGGAT	CTTGCATG	ATGATAAGGGTGAAT
p*rocDEF*	CTTGATT	TGGCA	CAGAA	CTTGCATT	TATATAAAGGGAAAG
p*bkd*	TAAGAGC	TGGCA	TGGAA	CTTGCATA	ATAAAAGGCGGAGTC

FIG. 3. Nucleotide sequences of promoter regions of the *acoABCL* operon (p*acoA*), the levanase operon (p*lev*) (6), the *rocABC* operon (p*rocABC*) (4), the *rocG* gene (p*rocG*) (3), the *rocDEF* operon (p*rocDEF*) (14), and the *bkd* operon (p*bkd*) (8). The transcriptional start sites are indicated by arrows. The boxes indicate conserved DNA sequences around positions −12 and −24 with respect to the transcriptional start sites.

operon. This possibility was tested by constructing a *B. subtilis* strain in which the *lacZ* gene was integrated and fused into the *acoR* gene. A DNA fragment encoding an internal part of *acoR* was obtained by PCR synthesis and inserted into pDIA5307 upstream from the *lacZ* gene. The recombinant plasmid was integrated by a single-crossover event into the chromosome of strain 168, leading to strain QB7713. As the integration of the *lacZ* gene inactivates the *acoR* gene, the plasmid pACOR1, which contains the wild-type *acoR*, was introduced by transformation into strain QB7713, leading to strain QB7714. The level of *lacZ* expression was assayed in extracts of cultures of strains QB7714 and QB7713 grown in CSK medium containing 10 mM acetoin (Table 2). The results indicate that the transcription of *acoR* was not induced by acetoin and did not require the wild-type *acoR* gene for its expression. Therefore, the *acoR* gene and the *acoABCL* operon are organized as two distinct transcriptional units and *acoR* is not autoregulated. In addition, the transcription of *acoR* is strongly repressed by the presence of glucose in the growth medium. A Δ*ccpA*::*spc* null mutation was introduced by transformation into strain QB7714, leading to strain QB7733 (Table 1). The properties of this strain show that the repression by glucose of the *acoR* transcription depended upon CcpA (Table 2).

Catabolite repression of *aco* operon expression. The *acoABCL* operon of *B. subtilis* is subject to carbon catabolite repression by glucose *via* CcpA (Table 2). Several CRE-like sequences could be involved in carbon catabolite repression by glucose. A DNA sequence, 5′-TGATTTTACGGGCTCA-3′, is located between positions −34 and −49 from the transcription start site of the *acoABCL* operon. Another DNA sequence, 5′-TGAATTCGGTCCCA-3′, is located in the beginning of the coding sequence of *acoR* (codons 1 to 5). Mutants were constructed containing either point mutations or deletions of these CRE-like sequences. The glucose effect in the resulting strains was assayed and indicated that neither CRE-like sequence is involved in catabolite control by glucose (not shown). Two other CRE-like sequences are located in the intergenic region between *acoL* and *acoR* (Fig. 5). In order to test the binding of CcpA in the upstream region of *acoR*, DNase I footprinting experiments were performed. A 239-bp DNA fragment containing both CRE-like sequences located



Strain	β-Galactosidase activity (Miller units. / mg protein)	
	CSK	CSK + 10mM acetoin
QB7700 (ΔA)	165	29660
QB7719 (ΔB)	200	27655
QB7720 (ΔC)	215	28030
QB7721 (ΔD)	25	310

FIG. 4. Nucleotide sequence of the *acoA* upstream region. The deletion end points are indicated by bent arrows and numbered with respect to the transcriptional start site, labeled with an asterisk. −12, −24 sequences are indicated. The boldly underlined regions indicate putative palindromic UAS. The effects of upstream deletions on expression of the *acoA′-′lacZ* translational fusion are indicated on the right. β-Galactosidase specific activity was determined in extracts prepared from exponentially growing cells in CSK medium containing 10 mM acetoin as the inducer.

between the end of *acoL* and the beginning of *acoR* was used as a template. The results of the DNase I footprinting experiments are presented in Fig. 6. In the presence of CcpA, a clear protection pattern was observed in a single region extending from 45 to 71 bases upstream from the ATG start codon of *acoR*. A specific interaction between CcpA and HPr-Ser-P has been demonstrated by several in vitro techniques (19). Sites of hypersensitivity to DNase I digestion were observed when CcpA and HPr-Ser-P were present in the reaction mixture. Similar alterations of the pattern of DNase I digestion were previously observed outside the CRE sequence in other systems (31).

DISCUSSION

B. subtilis cells growing in medium containing an excess of carbon source excrete a large number of organic compounds, including pyruvate, succinate, acetate, and acetoin. It was previously proposed that the conversion of pyruvate to acetoin or butanediol prevents overacidification of the growth medium during exponential growth (16). Acetoin is secreted during the exponential growth phase and also serves as a carbon storage compound which can be reused when other carbon sources have been exhausted. It is interesting to note that the CcpA protein is required for the expression of the *als* gene, involved in the biosynthesis of acetoin, and of the *ack* and *pta* genes, which are involved in the production of acetate. Conversely, CcpA is a repressor of the transcription of the *acu* and *aco* operons. *B. subtilis* therefore has a fine-tuning system controlling both the synthesis and the degradation of secondary metabolites, such as acetate and acetoin. We studied the regulation of transcription of the *aco* operon. It contains four genes, *acoABCL*, encoding the acetoin dehydrogenase complex. Transcription of this operon is strongly induced in the presence of acetoin in the growth medium. A regulatory gene, called *acoR*, is required for the positive regulation of this operon. This gene encodes a positive regulator containing a central domain which is probably involved in the activation of the −12, −24 promoter. In this family of transcriptional activators, there are several domains with different functions. Typically,

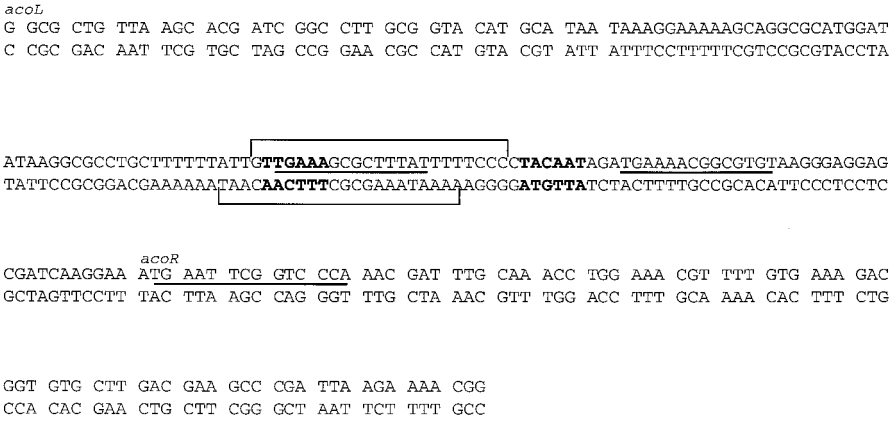


FIG. 5. Organization of the *acoR* promoter region. The DNA sequence located between the end of *acoL* and the beginning of *acoR* is shown. DNA regions with similarities to the CRE consensus sequence are underlined. Putative −10 and −35 regions of the *acoR* promoter are indicated in boldface letters. Regions protected by CcpA are marked by brackets.

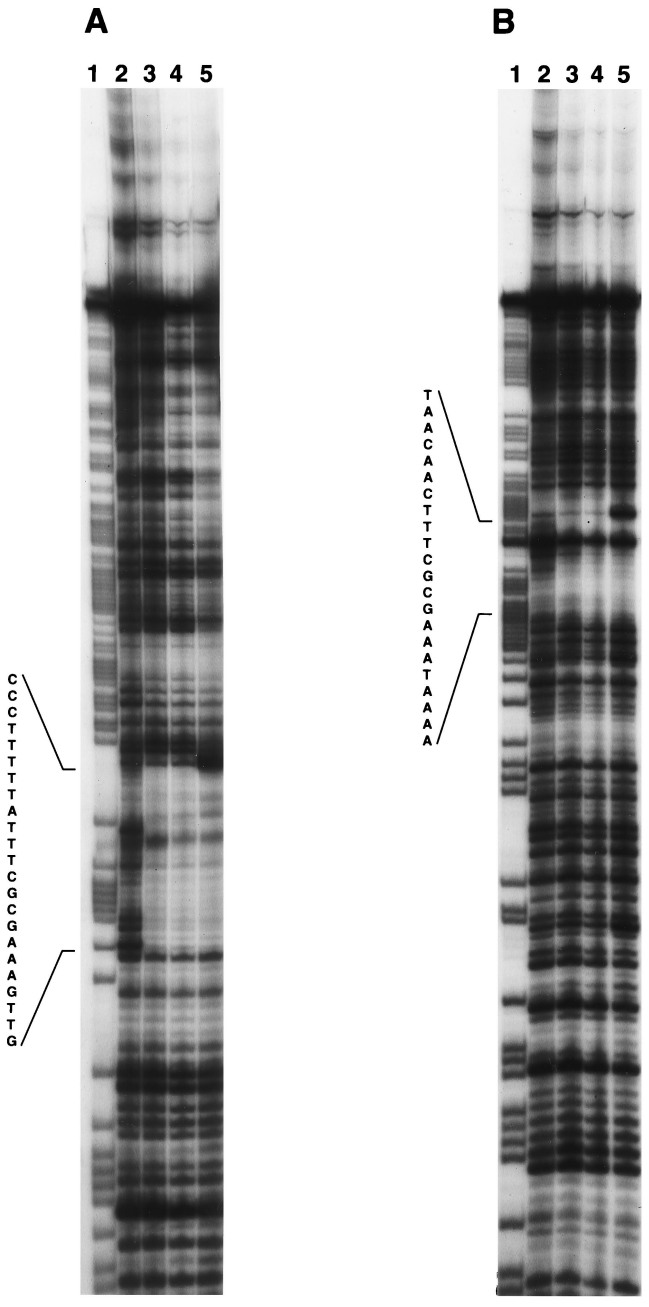


FIG. 6. DNase I footprinting analysis of CcpA binding to the *acoR* promoter region. Lanes containing 2×10^5 cpm of the labeled nontemplate strand (A) and template strand (B) of *acoR* are shown. Fragments were incubated in the presence of 2 μM purified CcpA. A + G Maxam and Gilbert reaction products of the appropriate DNA fragments were loaded in lanes 1. Lanes 2 through 5 were as follows: lanes 2, no protein; lanes 3, 2 μM CcpA; lanes 4, 2 μM CcpA and 10 μM HPr; and lanes 5, 2 μM CcpA and 10 μM HPr-Ser-P. Regions protected by CcpA are indicated by DNA sequences.

the amino-terminal domain is the signal reception domain. The amino-terminal part of AcoR is a large domain of 300 residues, a characteristic which is shared with AcoR from *A. eutrophus* (21) and BkdR from *B. subtilis* (8). This amino-terminal domain is probably involved in the control of AcoR activity in response to the presence of the internal inducer. We also characterized a DNA sequence located between positions −85 and −123 with respect to the *acoA* transcription start site and demonstrated that it is involved in the transcription of the gene. This sequence contains a perfect palindromic structure of 2 × 11 bp. Since the upstream part of the palindromic structure is located between the deletion ΔC and the deletion ΔD, it is tempting to speculate that this sequence is the target of AcoR. Glucose represses the transcription of the *aco* operon, and this catabolic control depends upon the catabolite control protein A (CcpA). CcpA is a pleiotropic repressor and binds to *cis*-active operator sequences. An interaction of HPr-Ser-P with CcpA has been demonstrated in vitro, and the resulting complex binds specifically to the CRE sequence in several genes of *B. subtilis*. In addition to HPr, an HPr-like protein, Crh, exhibiting 45% identity with HPr, participates in catabolite repression (13). A DNA sequence located between positions −50 and −38 in the promoter of the levanase operon shows similarities to the CRE sequence. This sequence plays a role in catabolite repression of the *lev* operon (30, 31). Interestingly, there is a similar DNA sequence at approximately the same position in the promoter region of *acoA*. Point mutations introduced by site-directed mutagenesis did not affect repression by glucose. The transcription of *acoR* itself is repressed by glucose, and a DNA sequence located upstream from the start codon of *acoR* is protected by CcpA against DNase I digestion. This protected region contains the sequence 5′-TGAAAGCG CTTTAT-3′, which matches the CRE consensus sequence proposed by Weickert and Chambliss (41) except for the last 2 bases. Similar deviations from the CRE consensus sequence were previously observed in other systems (19). Sites of hypersensitivity to DNase I digestion were observed when HPr-Ser-P was present in the reaction mixture, suggesting that binding of CcpA and HPr-Ser-P might induce changes in the DNA structure. The protected region contains the sequence 5′-TGAAA GCGCTTTAT-3′, which matches the CRE consensus sequence. The protected sequence overlaps a 5′-TTGAAA-3′ sequence which is the presumed −35 sequence of the *acoR* promoter. Therefore, we conclude that repression of transcription of *acoR* is probably mediated by the binding of CcpA to the promoter region of *acoR*. It is likely that most if not all the catabolite repression of acetoin utilization by glucose in *B. subtilis* can be attributed to the control of transcription of *acoR* by CcpA.

ACKNOWLEDGMENTS

We thank Isabelle Martin-Verstraete for helpful discussion, Anne Galinier for the gift of CcpA, HPr, and HPr-Ser-P, Alex Edelman for correcting the manuscript, and Christine Dugast for expert secretarial assistance.

This work was supported by research funds from the Institut Pasteur, the Centre National de la Recherche Scientifique, and the Ministère de la Recherche. Naima Ould Ali is the recipient of a grant from the French Ministry of Cooperation and the Algerian Ministry of Higher Education and Scientific Research.

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