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Regulation of the *Bacillus subtilis alsS*, *alsD*, and *alsR* Genes Involved in Post-Exponential-Phase Production of Acetoin†

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Acetoin is a major extracellular product of *Bacillus subtilis* grown on glucose and other fermentable carbon sources. The enzymes responsible for the formation of acetoin, acetolactate synthase, and acetolactate decarboxylase are synthesized in detectable amounts only in cells that have reached stationary phase. We have cloned and sequenced the genes encoding these enzymes, *alsS* and *alsD*, as well as a gene, *alsR*, that regulates their expression. *alsS* and *alsD* appear to compose a single operon, while *alsR* is transcribed divergently from the *alsSD* operon. AlsR shows significant homology to the LysR family of bacterial activator proteins, and when *alsR* is disrupted the *alsSD* operon is not expressed. Transcriptional fusions to *alsS* and *alsR* revealed that AlsR is required for the transcription of the *alsSD* operon, which increases during stationary phase. Two mutations that cause increased expression of the *alsSD* operon have been isolated, cloned, and sequenced. They each change an amino acid in the AlsR protein.

There is a single acetohydroxy acid synthase (AHAS) in *Bacillus subtilis*, the product of the *ilvBN* genes. It catalyzes the condensation of an activated aldehyde moiety derived from pyruvate with a second molecule of pyruvate to form acetolactate, a precursor of valine, or with α-ketobutyrate to form α-acetohydroxybutyrate, a precursor of isoleucine. Strains of *B. subtilis* that carry a deletion of the *ilvB* gene require exogenous isoleucine and valine for growth on minimal medium (41).

There is a second pathway for acetolactate synthesis in *B. subtilis*, however. The *alsS* gene encodes α-acetolactate synthase, an enzyme involved in the production of acetoin in stationary-phase cultures of *B. subtilis* (41). AlsS condenses two molecules of pyruvate to form acetolactate. Acetolactate is converted to acetoin by spontaneous decarboxylation at low pH and by the action of acetolactate decarboxylase (16). Acetolactate decarboxylase (AlsD) in *B. subtilis* is encoded by the *alsD* gene. Acetoin can then be excreted by the cells. It has been suggested that acetoin production is a mechanism for maintaining the internal pH of cells that have entered the stationary phase and accumulate pyruvic acid that is not required for biosynthesis. In *Lactobacillus plantarum* (27) acetoin production appears to assist internal pH maintenance.

AlsS is synthesized in detectable quantities only in stationary-phase cultures (41) (see Results). However, Holtzclaw and Chapman (11) have shown that AlsS activity is inducible during logarithmic growth by addition of high concentrations of acetate to the growth medium.

We have found mutations that give rise to expression of AlsS in the exponential phase (41). These mutations were isolated in a strain of *B. subtilis* that lacked the IlvB protein, required to carry out the first common step in the biosynthesis of isoleucine and valine (28). They result in *ilvBΔ1* strains that grow on minimal medium in the absence of added valine but still require isoleucine.

This paper reports the cloning and sequencing of *alsS*, *alsD*, and *alsR*. We show that *alsS* and *alsD* probably compose a single operon and that transcription through this operon is increased at the onset of stationary phase. Disruption of *alsR* results in the loss of expression of *alsS* and prevents transcription of the *alsSD* operon. AlsR is homologous to the LysR family of activator proteins (9), and *alsR* is divergently transcribed from a promoter very close to the promoter of the *alsSD* operon. At least two of the cloned mutations that result in increased expression of *alsS* lie in the *alsR* gene and alter different amino acids in the AlsR protein. These alleles are called *alsR1* and *alsR8*.

In addition, *ccpA/alsA*, a gene required for the catabolite repression of the stationary-phase degradative α-amylase gene (10), is required for the transcription of *alsS*. A strain carrying the *alsR1* mutation as well as a mutation of *alsA* (*alsA1*) does not express *alsS*. *alsA1* appears to be a null mutation; the phenotype of strains carrying *alsA1* is identical to that of a strain that carries a Tn*917* insertion in *ccpA/alsA*.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in this study are described in Table 1. *Escherichia coli* JM2r$^-$ and JM109 were used for all plasmid constructions.

Media and bacterial growth. Complex agar medium was tryptose blood agar base (Difco). Minimal agar medium contained Spizizen minimal salts (GIBCO), 0.5% glucose, 10 μg of tryptophan per ml, 3 mg of NH_4Cl per ml, 10 μM $MnCl_2$, 1 μg of biotin per ml, and 1.7% Noble agar (Difco). Minimal medium was supplemented with the appropriate amino acids (40 μg/ml). Bacteria were grown in Luria-Bertani (LB) broth. Antibiotics were used for *B. subtilis* at the following concentrations: 5 μg/ml for chloramphenicol; 1 μg/ml for erythromycin plus 25 μg/ml for lincomycin. *E. coli* transformants were selected on 25 or 50 μg of ampicillin per ml and 25 μg of chloramphenicol per ml.

Transformation and transduction. Competent cells of *B. subtilis* were prepared and transformed as described in reference 38. *E. coli* transformations were carried out by the method of Hanahan (7), substituting LB broth as the growth medium for SOC; the dithiothreitol step was omitted. PBS1

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TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype	Source or reference
B. subtilis		
CU371	*ilvBΔ1 trpC2*	41
CU532	*ilvBΔ1 trpC2 alsR1 alsA1*	41
CU845	*ilvBΔ1 trpC2 alsR1 hisA1*	41
CU3726	*zih83*::Tn*917 trpC2*	This study
CU4175	*zih83*::Tn*917*[21Δ2] *trpC2*	This study
CU4769	*ilvBΔ1 trpC2 alsR*::*cat*	This study
CU4784	*ilvBΔ1 trpC2 alsS*::*cat*	This study
CU4800	*ilvBΔ1 trpC2 amyE*::*alsS-lacZ*	This study
CU4802	*ilvBΔ1 trpC2 alsR1 amyE*::*alsS-lacZ*	This study
CU4804	*ilvBΔ1 trpC2 alsR1 alsA1 amyE*::*alsS-lacZ*	This study
CU4810	*ilvBΔ1 trpC2 alsR*::*cat amyE*::*alsS-lacZ*	This study
CU4930	*ilvBΔ1 trpC2 alsR1 alsD*::*cat*	This study
CU4955	*ilvBΔ1 trpC2 alsR8 amyE*::*alsS-lacZ*	This study
E. coli		
CU9090	*rbs221 ilvB2102 ilvHI2202 pro*	H. Umbarger
JM2r$^-$	*mcrAB hsdRM*$^+$ *recA1* Δ(*lac-proAB*) *thi gyrA96 relA1 srl*::Tn*10* F′(*proAB lac*^{q}*ZDM15*)	M. Weiner
CU9305	*serB*::Tn*5 mcrAB* ϕ80 P2	P. B. Vander Horn
JM101	*hsdRM*$^+$ *recA1* Δ(*lac-proAB*) *thi gyrA96 relA1 srl*::Tn*10* F′(*proAB lac*^{q}*ZDM15*)	J. Messing

transductions of *B. subtilis* were performed as previously described (31).

DNA isolation. Chromosomal DNA was isolated from *B. subtilis* as described in reference 38, omitting the pronase step. Large-scale preparations of plasmids were obtained by boiling lysis and high-speed centrifugation in a cesium chloride gradient (19).

Cloning of *alsS* and *alsR*. The *alsR1* mutation maps to 314° on the *B. subtilis* chromosomal map (41). To clone *alsR*, a search for closely linked transposon Tn*917* insertions generated as described in reference 29 was undertaken. The Tn*917* insertion in CU3726 was cotransformed 85% with the *alsR1* mutation. The method described in reference 40 was used to clone the chromosomal DNA flanking Tn*917*. CU3726 was transformed with linearized pTV21Δ2 (containing a *cat* gene and an *E. coli* replication origin [39]), selecting chloramphenicol-resistant transformants that had lost macrolide-lincosamide-streptogramin B resistance. Chromosomal DNA from an appropriate transformant (CU4175) was isolated and cut with *Sph*I, diluted, ligated, and introduced into *E. coli*. This resulted in the cloning of a 3.7-kb fragment of the chromosome on an *E. coli* plasmid named pDK6 (Fig. 1).

The *alsR1* allele was cloned by constructing a phage λ library of partial *Mbo*I-cut chromosomal DNA, isolated from CU845 (*ilvBΔ1 alsR1 hisA1*), cloned into *Bam*HI-cut λ-EMBL3 arms (from Promega). Recombinant phages were screened for inserts that hybridized to pDK6. One such phage, λF, showed strong hybridization to pDK6 and carried about 17 kb of *B. subtilis* chromosomal DNA. DNA isolated from the phage was capable of transforming CU371 (*ilvBΔ1 alsR*$^+$) to the *alsR1* phenotype. *Eco*RI fragments of this phage were cloned into pUC19 (37) and tested for their ability to transform CU371 to valine prototrophy. A 6.6-kb *Eco*RI fragment in plasmid pMR110 was shown to carry the *alsR1* mutation, and a 2.3-kb *Hin*dIII subclone in pUC19, designated pMR115, was found to transform CU371. An equivalent subclone of the 2.3-kb *Hin*dIII fragment from pDK6 (*alsR*$^+$) into pUC19 was constructed and designated pMR215. These plasmids are shown in Fig. 2.

The *alsR8* allele was cloned in the same manner as *alsR*$^+$.

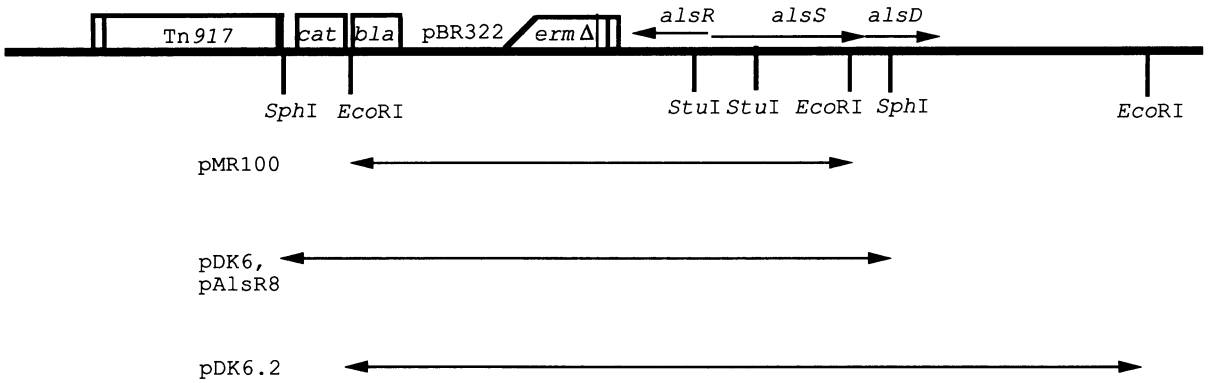


FIG. 1. Structure of Tn*917*[21Δ2] and the plasmids isolated from strains carrying the Tn*917* replacement. The Tn*917* insertion was replaced by pTV21Δ2 linearized with *Pst*I to introduce the *E. coli* origin of replication and β-lactamase gene into the transposon, allowing the cloning of the sequences flanking the transposon. pDK6 carries 3.7 kb of flanking DNA, and pDK6.2 carries 7.2 kb of flanking DNA from strains wild-type for *alsR*. pAlsR8 contains the *alsR8* allele. pMR100 was constructed by cutting pDK6 with *Eco*RI and religating the result to delete the *cat* gene.

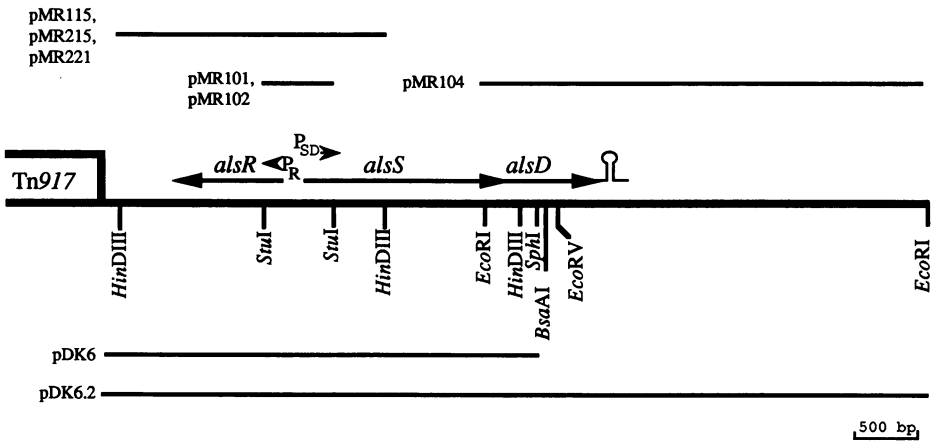


FIG. 2. Restriction map of the *alsR*, *alsS*, and *alsD* genes of *B. subtilis*. The location and direction of transcription of the coding regions are indicated by the arrows. Putative promoters (P_{SD} and P_R) for each of the operons are shown as arrowheads, and the *alsSD* terminator is indicated by the stem-loop at the end of the transcription unit. The transposon Tn*917* insertion used to clone the region from the chromosome is shown. The lines below the map show the extent of the sequences that were cloned in pDK6 and pDK6.2. Restriction sites used for cloning and for disruption of the *alsS*, *alsR*, and *alsD* genes are shown below the map. The lines above the map show the extent of the region subcloned in the plasmids shown. pMR101 and pMR102 carry the 700-bp *Stu*I fragment containing the intergenic region cloned in both orientations into pUC19. pMR115 carries the *alsR1* allele; pMR221 carries the *alsR8* allele.

Plasmid pA1sR8 is identical to pDK6 (Fig. 1) except that it carries the *alsR8* allele. A 2.3-kb *Hin*dIII fragment containing *alsR8* was subcloned into pUC19 to generate pMR221 (Fig. 2).

Cloning of *alsD*. The *alsD* gene was cloned from phage λF. Sequencing of the 3.7-kb insert of pDK6 revealed a region of the insert that was homologous to the amino terminus of the acetolactate decarboxylases of *Enterobacter aerogenes* (25) and *Bacillus brevis* (2). The homology extended to the *Sph*I site used to clone pDK6, and we believed that the open reading frame (ORF) extended beyond this site. Restriction analysis of pDK6 revealed an *Eco*RI site in *alsS*, 423 bp upstream of the *Sph*I site (Fig. 1 and 2). The 423-bp *Eco*RI-*Sph*I fragment was used as a probe to isolate the overlapping *Eco*RI fragment from λF. Southern blots of *Eco*RI-cut λF showed that the next *Eco*RI site was 3 kb downstream of the *Sph*I site. Plasmid pMR104 (Fig. 2) was constructed by cloning the 3.5-kb *Eco*RI fragment from λF into pUC19 and was shown to carry *alsD* by sequence analysis.

Plasmid pDK6.2 was generated by partially digesting chromosomal DNA from CU4175 (*ilvBΔ1* Tn*917*[21Δ2]) with *Eco*RI. It carries flanking *B. subtilis* DNA that extends from the transposon to the *Eco*RI site downstream of *alsD* (Fig. 1).

Disruption of *alsS* and *alsR*. There are a single *Stu*I site in the coding region of *alsS* and a single *Stu*I site in the coding region of *alsR* (Fig. 1 and 2). pDK6 carries a *cat* gene derived from pTV21Δ2. It is flanked by *Eco*RI sites, one in *alsS* and the other in pTV21Δ2. pMR100 (Fig. 1) was constructed by cutting pDK6 with *Eco*RI, diluting it, and religating the result. This removed the *cat* gene from pDK6 while retaining the ampicillin resistance gene. This allowed us to inactivate both *alsS* and *alsR* with a *cat* gene derived from pTV54.

A 1.5-kb fragment carrying a *cat* gene was obtained by cutting pTV54 (39) with *Sma*I and gel purifying the desired fragment. pMR100 was partially digested with *Stu*I and subjected to ligation with the *cat* gene.

Restriction analysis was performed on plasmids isolated from chloramphenicol-resistant transformants to determine which gene had been disrupted. Also, the *E. coli* strain into which the ligation was transformed could be tested for acetoin production (15). *E. coli* transformed with pDK6 or pMR100 produces acetoin. It was possible to determine which plasmids no longer allowed acetoin formation; such plasmids were shown to carry a disrupted *alsS* gene. This suggests that *alsS* is expressed in *E. coli* in the absence of the *alsR* gene product. This may be due to readthrough from a promoter on the plasmid or to initiation of transcription from sequences upstream of *alsS* by the *E. coli* RNA polymerase. pMR100R carries a disrupted *alsR* gene, and pMR100S carries a disrupted *alsS* gene.

Construction of pMR111. To facilitate isolation of the *cat* gene for gene disruption, plasmid pMR111 was constructed. Plasmid pTV54 was cut with *Sma*I, and the 1.5-kb fragment carrying the *cat* gene was gel purified. This fragment was cloned into pUC19 cut with *Sma*I to generate pMR111.

Disruption of *alsD*. Repeated attempts to disrupt *alsD* by introducing the *cat* gene from pTV54 into the *alsD* gene cloned in plasmid pMR204 failed, so an internal *Hin*dIII-*Eco*RV fragment of *alsD* (nucleotides 3837 to 4399) (Fig. 2) was cloned into pUC19 to construct plasmid pMR120. pMR120 was linearized with *Bsa*AI, which has a single target within the fragment of *alsD* carried in pMR120. The *cat* gene was gel purified from pMR111 cut with *Sma*I and ligated to pMR120/*Bsa*AI to construct pMR121. Plasmid pMR121 was isolated from chloramphenicol-resistant transformants of *E. coli* and shown to carry two *cat* genes cloned in tandem in the *Bsa*AI site within the *alsD* fragment. pMR121 was cut with *Nco*I, for which there is a single site in each *cat* gene, and the cut DNA was diluted and religated. This resulted in the construction of plasmid pMR122, which carried a single *cat* gene cloned into the *Bsa*AI site within *alsD*.

alsD on the chromosome was disrupted by linearizing pMR122 with *Sca*I and transforming CU4768 (*ilvBΔ1 alsR1*) to chloramphenicol resistance, constructing strain CU4930.

Verification of the disruptions of *alsS*, *alsR*, and *alsD* in the chromosome. The polymerase chain reaction was employed to prove the disruptions of *alsS*, *alsR*, and *alsD* in the chromosome. Primers flanking the sites of insertion in each

gene were used to amplify that region of the gene from strains carrying the wild-type and disrupted alleles.

For the *alsS* knockout, the following primers were used: primer S1, which anneals to a sequence upstream of the *Stu*I site in *alsS* (nucleotides 1938 to 1952), and primer S2, which anneals to a sequence downstream of the *Stu*I site (nucleotides 2349 to 2335). In strains wild type for *alsS*, these primers should generate a 411-bp polymerase chain reaction product. A strain carrying *alsS*::*cat* should lack the 411-bp product, and instead a product of approximately 2 kb should be amplified. DNA from strains CU371, CU4769, and CU4930 (*alsS*$^+$) produced only the 411-bp polymerase chain reaction product, and CU4784 (*alsS*::*cat*) DNA lacked the 411-bp product but generated a 2-kb product.

In a similar fashion, primer R2, which anneals to a sequence upstream of the *Stu*I site (nucleotides 1484 to 1498), and primer R5, which anneals to a sequence downstream of the *Stu*I site (nucleotides 1103 to 1090), were used to demonstrate that the *alsR* gene was disrupted in strain CU4769. These primers amplified the expected 408-bp product from strains wild type for *alsR* (CU371, CU4784, and CU4930) and amplified only a 2-kb fragment from the strain carrying *alsR*::*cat* (CU4769).

Primer D2, which anneals to a sequence upstream of the *Bsa*AI site in *alsD* (nucleotides 3850 to 3868), and primer D102, which anneals to a sequence downstream of the *Bsa*AI site (nucleotides 4393 to 4374), were used to demonstrate that the *alsD* gene was disrupted in strain CU4930. These primers amplified the expected 543-bp product from strains wild type for *alsD* (CU371, CU4769, and CU4784) and amplified only a 2.1-kb fragment from the strain carrying *alsD*::*cat* (CU4930).

The presence of only the expected products in the DNA from each strain and their subsequent sequencing verified that the above strains carried disrupted alleles in their chromosomes (unpublished experiments).

***lacZ* plasmid and strain constructions.** The plasmid for assaying the transcription of *alsS* was constructed by introducing the *alsR-alsSD* intergenic region into plasmid pDH32 (provided by Dennis Henner). pDH32 carries a promoterless *lacZ* gene downstream of a multiple cloning site and a *cat* gene flanked by the right and left halves of the *amyE* gene. The 700-bp *Stu*I fragment (Fig. 1 and 2) from pDK6, containing the divergent promoters, was cloned in both orientations into pUC19 cut with *Sma*I, to construct plasmids pMR101 and pMR102 (Fig. 2). Plasmid pMR102 was cut with *Eco*RI and *Bam*HI, which flank the *Sma*I site, and the promoter-containing fragment was cloned into pDH32 cut with *Eco*RI and *Bam*HI to construct plasmid pMR106 (Fig. 3).

The *lacZ* fusion was introduced into the chromosome of *B. subtilis* by linearizing pMR106 with *Sca*I and transforming competent cells, selecting for chloramphenicol resistance. The resulting transformant (CU4800) contains the *alsS-lacZ* fusion located in single copy within the unlinked *amyE* gene (36). To confirm that the fusion resides within the *amyE* gene, a PBS1 lysate made on CU4800 was used to transduce CU1182 (*trpC2 aroI906 dal-1 purB33*) to chloramphenicol resistance. Linkage to *aroI906* (31°) was demonstrated, indicating that the *lacZ* fusion was located within the *amyE* gene (29°). The fusion has been moved into various genetic backgrounds by PBS1 transduction.

To construct strains to examine the transcription of *alsS* in an *alsR* mutant background, *alsR*::*cat* was introduced into CU4800 by cotransduction with the Tn*917* from CU3726. Transductants resistant to the antibiotics erythromycin and

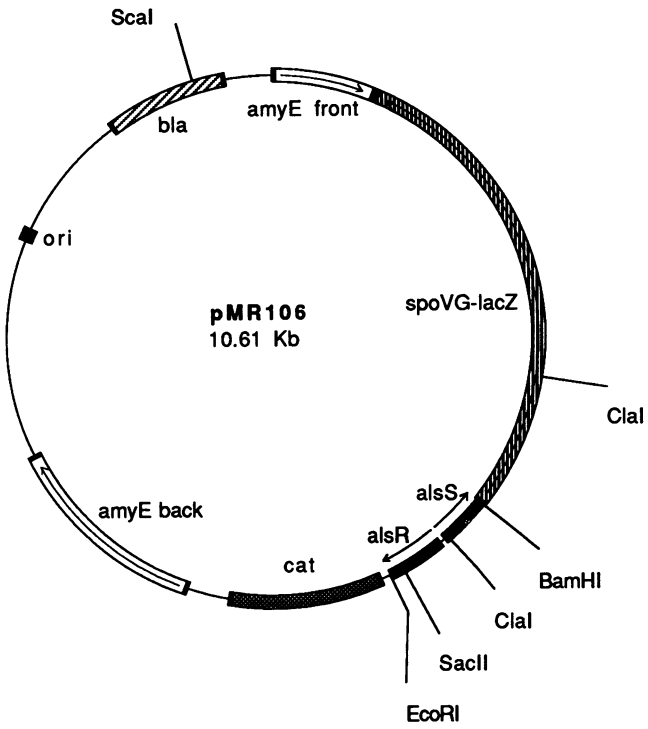


FIG. 3. Construction of pMR106 for analyzing transcription of *alsS*. pMR102 (Fig. 2) was cut with *Eco*RI and *Bam*HI, and the fragment carrying the divergent promoters was cloned into pDH32 cut with *Eco*RI and *Bam*HI to construct pMR106. The orientation of the promoter-containing fragment in pMR102 was such that this generated a fusion between *alsS* and *lacZ*.

lincomycin (macrolide-lincosamide-streptogramin B resistant) were tested to determine whether they carried the disrupted allele of *alsR* in the following manner: *ilvBΔ1 alsR*$^+$ strains of *B. subtilis* will grow on minimal medium plus isoleucine if the plates are supplemented with 100 mM potassium acetate, but similar strains lacking *alsR* will not (see Results).

DNA sequencing. Nucleotide sequences were determined by the dideoxy chain termination method of Sanger et al. (24). Denatured plasmid DNA and overlapping single-stranded recombinant phage M13 clones served as templates. All sequencing reactions were performed by using Sequenase 2.0 as specified by the manufacturer (United States Biochemical Corp.) for either single-stranded or double-stranded sequencing, and all regions were sequenced in both directions. Where necessary, dITP or 7-deaza-GTP was used in place of dGTP to reduce compression artifacts. Primers used were either the M13 forward and reverse primers or those synthesized by the Analytical and Synthetic Facility of Cornell University.

Computer-assisted sequence analysis. Protein comparisons were obtained by searching the GenPept library, using FASTA (23).

β-Galactosidase assays. Cells containing transcriptional fusions were grown in LB broth plus 0.5% glucose, and 1-ml samples were collected at various time points in logarithmic and stationary phases, frozen in dry ice, and stored at −20°C. The samples were thawed, and cell density was measured at 600 nm. Depending on the expected activity of the sample, some fraction of the sample was centrifuged and

the cells were resuspended in 0.5× PM2 buffer (37 mM NaH_2PO_4, 63 mM Na_2HPO_4, 1 mM $MgSO_4$, 1 mM $MnSO_4$, and 100 mM β-mercaptoethanol) plus 0.01% deoxycholate and 0.1 mg of hexadecyltrimethylammonium bromide per ml. *ortho*-Nitrophenylgalactoside was added, and the cells were incubated at 28°C. The cleared supernatant of each sample was assayed for specific activity by the method of Miller (20).

RNA isolation and primer extension. Cells were grown in minimal medium supplemented with tryptophan, isoleucine, and valine. Potassium acetate (40 mM) was added to some of the cultures. Samples were harvested at points late in the exponential phase and 1 h into stationary phase. RNA isolation was performed as described in reference 5. Primer extension was carried out by modifying a protocol provided by David Stern (Cornell University). Reactions were performed as follows: 10 μg of RNA, 4.5 ng of end-labelled primer, 0.5 U of RNase Block II (from Stratagene), and 0.7 mmol of each deoxynucleoside triphosphate were mixed in a 10-μl volume. This was incubated for 5 min at 75°C and then for 5 min at 50°C before 12.5 U of avian myeloblastosis virus reverse transcriptase (Molecular Genetics Resources Inc.) was added. The reaction mixture was incubated at 50°C for 15 min before the reaction was stopped by addition of sequencing stop buffer.

Voges-Proskauer tests. Overnight cultures in LB broth were diluted into Penassay antibiotic medium 3 plus 0.5% glucose in Klett flasks and were then grown to various points in the exponential and stationary phase. One-milliliter samples were removed, and to them 0.6 ml of 5% α-naphthol in 100% ethanol and 0.2 ml of 40% KOH-creatine were added. Tubes were shaken vigorously after addition of the reagents. Results were read no later than 4 h after addition of reagents.

Enzymes and reagents. All restriction enzymes were purchased from and used under the conditions specified by commercial suppliers. T4 DNA ligase was purchased from Amersham International. Nested deletions of the recombinant M13 phage clones were generated with the Erase-a-base kit provided by Promega.

Nucleotide sequence accession number. The GenBank/EMBL accession number for the *alsR-alsSD* sequence presented in this paper is L04470.

RESULTS

Isolation of the *alsS*, *alsR*, and *alsD* genes. As described above, strains of *B. subtilis* that are deleted for the *ilvB* gene can mutate to valine prototrophy while remaining isoleucine auxotrophs. Such mutants express acetolactate synthase constitutively. The mutations that permitted constitutive expression of *alsS* were called *alsR* mutations (41).

Plasmid pDK6, which carries a 3.7-kb fragment from the *alsR* region of the *B. subtilis* chromosome (see Materials and Methods), permitted the growth of an *E. coli* strain, CU9090, lacking its endogenous AHAS isozymes (and which was therefore auxotrophic for isoleucine and valine) on minimal medium plus isoleucine in the absence of valine. This indicated that pDK6 carried *alsS*, the gene encoding acetolactate synthase (AlsS). Sequence analysis (described below) showed that the 3.7-kb *B. subtilis* fragment also carried *alsR*.

Plasmid pMR104 (see Materials and Methods) was used to determine the sequence of *alsD*. The organization of the genes in the divergent operons is shown in Fig. 2.

Nucleotide sequencing and analysis. The combined sequences of the overlapping inserts in pDK6 and pMR104 are shown in Fig. 4, as are the ORFs and their corresponding amino acid sequences. Analysis of the 3,702 bp of the *B. subtilis* insert in pDK6 identified two ORFs, each preceded by a sequence similar to the ribosome-binding sites seen in gram-positive bacteria (21). One ORF, designated AlsS, is 541 amino acids long and is predicted to encode a protein of 59.4 kDa. Computer-assisted comparisons of the amino acid sequence predicted by this ORF revealed that it is homologous to the IlvB protein of *B. subtilis* (28) and to the three AHAS isozymes of *E. coli* (35). AlsS is 22.9% identical to IlvB and 26.9% identical to AHAS I of *E. coli* (Fig. 5). A sequence that resembles a thiamine PP_i-binding site (8) was found in AlsS near the carboxyl terminus; a similar sequence was found at a homologous position in the other proteins; the presence of this putative site is consistent with the mechanism of acetolactate formation (4, 17). Thiamine PP_i has been shown to be required by the acetolactate synthase of *Serratia marcescens* (18), and the acetolactate synthase of *Aerobacter aerogenes* has been shown to bind TPP (14).

An ORF, designated AlsD, that begins 64 nucleotides downstream of the end of the *alsS* gene is predicted to encode a polypeptide 256 amino acids long. A possible transcription terminator with a predicted ΔG of −29 kcal/mol (ca. −120 kJ/mol) was found immediately downstream of *alsD*. A comparison of the predicted amino acid sequence of AlsD with those of the *B. brevis* and *E. aerogenes* acetolactate decarboxylases is shown in Fig. 6. Extensive amino acid sequence homology among the three proteins is seen; AlsD is 31% identical to the AldC of *B. brevis* and is 39% identical to the AldC of *E. aerogenes*.

Another ORF is transcribed divergently from *alsS* and is 302 amino acids long. It is predicted to encode a protein of 33.2 kDa. A computer search revealed that this protein is similar to the LysR family of transcriptional regulators (9), of which LysR and IlvY of *E. coli* (26, 33), NodD of *Rhizobium meliloti* (12, 13), GltC of *B. subtilis* (1), and OccR of *Agrobacterium tumifaciens* (6, 30) are members. We have named this gene *alsR*. A comparison of AlsR with IlvY and GltC is shown in Fig. 7. Members of this family are very similar in size and show extensive amino acid similarity, particularly at their NH_2-terminal ends, where a helix-turn-helix motif is found (9). Figure 7 also shows that a helix-turn-helix (identified by the method of Dodd and Egan to calculate a score that measures the resemblance of any stretch of 20 residues to the helix-turn-helix DNA-binding motif [3]) was found in AlsR at the amino terminus, in a position homologous to that seen for the other members of this family.

Mapping the 5′ end of the *alsS* message and identification of the *alsS* promoter. *alsSD* and *alsR* form a divergent pair of operons. The 5′ end of the *alsS* message was mapped by primer extension (Fig. 8). Primer 106, which anneals to nucleotides 1937 through 1908 and whose 3′ end is 29 nucleotides downstream of the putative translation start, was used. A single prominent product of 205 nucleotides was seen in primer extensions of RNA isolated from CU4955 (*ilvBΔ1 alsR8 amyE::alsS-lacZ*) during the exponential and stationary phases in both the presence and absence of acetate. The start of transcription appears to be at an A residue 146 nucleotides upstream of the putative initiation codon. In addition, primer extension with RNA isolated during stationary phase from CU371 (*alsR*$^+$) generated the identical product (data not shown).

Just upstream of the transcript start are sequences that are similar to SigA-dependent promoters. These sequences conform in 3 and 5 of 6 residues to the SigA consensus −35 and

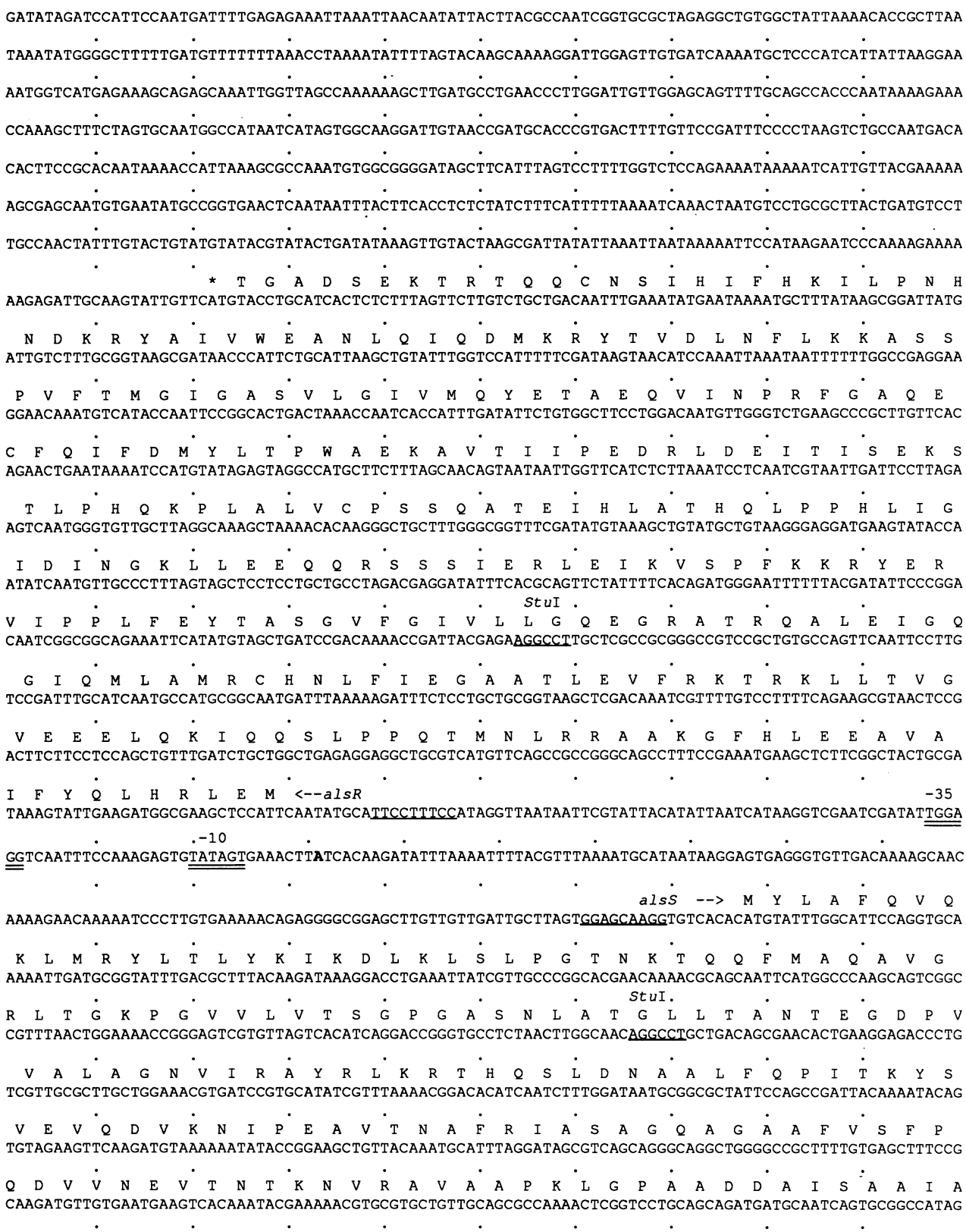
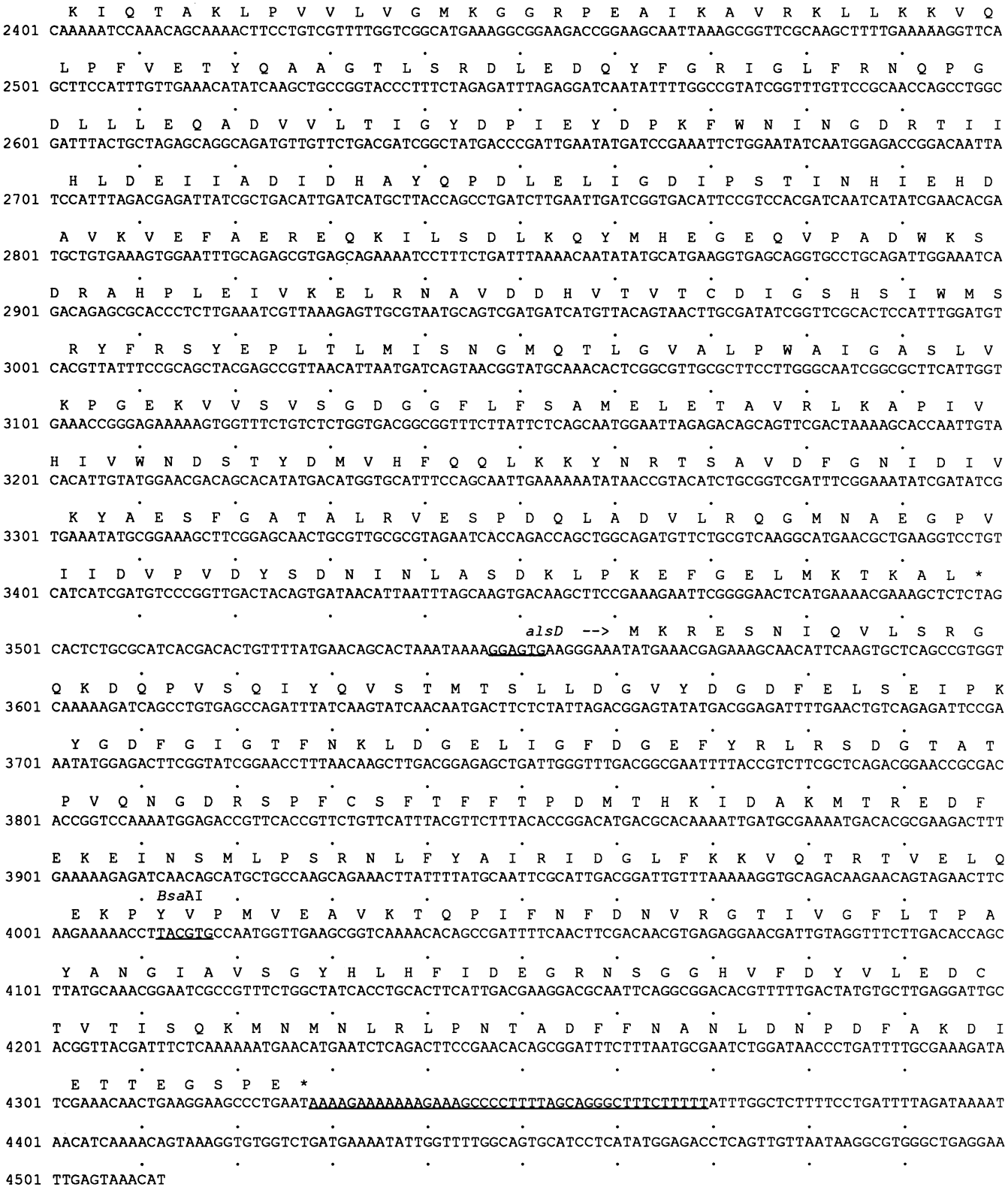


FIG. 4. Nucleotide sequence of the *alsSD* and *alsR* divergent operons. The sequence of the nontranscribed strand of the *alsS* and *alsD* genes and their corresponding amino acid sequences are shown, as are the sequence of the transcribed strand of the divergent *alsR* gene and its corresponding amino acid sequence. Restriction sites used to disrupt the three genes are shown above the sequence. Shine-Dalgarno sequences have been underlined, as has the putative terminator of the *alsSD* operon. The start site of transcription for *alsSD* is shown in boldface, and the putative promoter of *alsSD* is double underlined.


K I Q T A K L P V V L V G M K G G R P E A I K A V R K L L K K V Q
2401 CAAAAATCCAAACAGCAAAACTTCCTGTCGTTTTGGTCGGCATGAAAGGCGGAAGACCGGAAGCAATTAAAGCGGTTCGCAAGCTTTTGAAAAAGGTTCA
L P F V E T Y Q A A G T L S R D L E D Q Y F G R I G L F R N Q P G
2501 GCTTCCATTTGTTGAAACATATCAAGCTGCCGGTACCCTTTCTAGAGATTTAGAGGATCAATATTTTGGCCGTATCGGTTTGTTCCGCAACCAGCCTGGC
D L L L E Q A D V V L T I G Y D P I E Y D P K F W N I N G D R T I I
2601 GATTTACTGCTAGAGCAGGCAGATGTTGTTCTGACGATCGGCTATGACCCGATTGAATATGATCCGAAATTCTGGAATATCAATGGAGACCGGACAATTA
H L D E I I A D I D H A Y Q P D L E L I G D I P S T I N H I E H D
2701 TCCATTTAGACGAGATTATCGCTGACATTGATCATGCTTACCAGCCTGATCTTGAATTGATCGGTGACATTCCGTCCACGATCAATCATATCGAACACGA
A V K V E F A E R E Q K I L S D L K Q Y M H E G E Q V P A D W K S
2801 TGCTGTGAAAGTGGAATTTGCAGAGCGTGAGCAGAAAATCCTTTCTGATTTAAAACAATATATGCATGAAGGTGAGCAGGTGCCTGCAGATTGGAAATCA
D R A H P L E I V K E L R N A V D D H V T V T C D I G S H S I W M S
2901 GACAGAGCGCACCCTCTTGAAATCGTTAAAGAGTTGCGTAATGCAGTCGATGATCATGTTACAGTAACTTGCGATATCGGTTCGCACTCCATTTGGATGT
R Y F R S Y E P L T L M I S N G M Q T L G V A L P W A I G A S L V
3001 CACGTTATTTCCGCAGCTACGAGCCGTTAACATTAATGATCAGTAACGGTATGCAAACACTCGGCGTTGCGCTTCCTTGGGCAATCGGCGCTTCATTGGT
K P G E K V V S V S G D G G F L F S A M E L E T A V R L K A P I V
3101 GAAACCGGGAGAAAAAGTGGTTTCTGTCTCTGGTGACGGCGGTTTCTTATTCTCAGCAATGGAATTAGAGACAGCAGTTCGACTAAAAGCACCAATTGTA
H I V W N D S T Y D M V H F Q Q L K K Y N R T S A V D F G N I D I V
3201 CACATTGTATGGAACGACAGCACATATGACATGGTGCATTTCCAGCAATTGAAAAAATATAACCGTACATCTGCGGTCGATTTCGGAAATATCGATATCG
K Y A E S F G A T A L R V E S P D Q L A D V L R Q G M N A E G P V
3301 TGAAATATGCGGAAAGCTTCGGAGCAACTGCGTTGCGCGTAGAATCACCAGACCAGCTGGCAGATGTTCTGCGTCAAGGCATGAACGCTGAAGGTCCTGT
I I D V P V D Y S D N I N L A S D K L P K E F G E L M K T K A L *
3401 CATCATCGATGTCCCGGTTGACTACAGTGATAACATTAATTTAGCAAGTGACAAGCTTCCGAAAGAATTCGGGGAACTCATGAAAACGAAAGCTCTCTAG
alsD --> M K R E S N I Q V L S R G
3501 CACTCTGCGCATCACGACACTGTTTTATGAACAGCACTAAATAAAAGGAGTGAAGGGAAATATGAAACGAGAAAGCAACATTCAAGTGCTCAGCCGTGGT
Q K D Q P V S Q I Y Q V S T M T S L L D G V Y D G D F E L S E I P K
3601 CAAAAAGATCAGCCTGTGAGCCAGATTTATCAAGTATCAACAATGACTTCTCTATTAGACGGAGTATATGACGGAGATTTTGAACTGTCAGAGATTCCGA
Y G D F G I G T F N K L D G E L I G F D G E F Y R L R S D G T A T
3701 AATATGGAGACTTCGGTATCGGAACCTTTAACAAGCTTGACGGAGAGCTGATTGGGTTTGACGGCGAATTTTACCGTCTTCGCTCAGACGGAACCGCGAC
P V Q N G D R S P F C S F T F F T P D M T H K I D A K M T R E D F
3801 ACCGGTCCAAAATGGAGACCGTTCACCGTTCTGTTCATTTACGTTCTTTACACCGGACATGACGCACAAAATTGATGCGAAAATGACACGCGAAGACTTT
E K E I N S M L P S R N L F Y A I R I D G L F K K V Q T R T V E L Q
3901 GAAAAAGAGATCAACAGCATGCTGCCAAGCAGAAACTTATTTTATGCAATTCGCATTGACGGATTGTTTAAAAAGGTGCAGACAAGAACAGTAGAACTTC
*Bsa*AI
E K P Y V P M V E A V K T Q P I F N F D N V R G T I V G F L T P A
4001 AAGAAAAACCTTACGTGCCAATGGTTGAAGCGGTCAAAACACAGCCGATTTTCAACTTCGACAACGTGAGAGGAACGATTGTAGGTTTCTTGACACCAGC
Y A N G I A V S G Y H L H F I D E G R N S G G H V F D Y V L E D C
4101 TTATGCAAACGGAATCGCCGTTTCTGGCTATCACCTGCACTTCATTGACGAAGGACGCAATTCAGGCGGACACGTTTTTGACTATGTGCTTGAGGATTGC
T V T I S Q K M N M N L R L P N T A D F F N A N L D N P D F A K D I
4201 ACGGTTACGATTTCTCAAAAAATGAACATGAATCTCAGACTTCCGAACACAGCGGATTTCTTTAATGCGAATCTGGATAACCCTGATTTTGCGAAAGATA
E T T E G S P E *
4301 TCGAAACAACTGAAGGAAGCCCTGAATAAAAGAAAAAAAGAAAGCCCCTTTTAGCAGGGCTTTCTTTTTATTTGGCTCTTTTCCTGATTTTAGATAAAAT
4401 AACATCAAAACAGTAAAGGTGTGGTCTGATGAAAATATTGGTTTTGGCAGTGCATCCTCATATGGAGACCTCAGTTGTTAATAAGGCGTGGGCTGAGGAA
4501 TTGAGTAAACAT

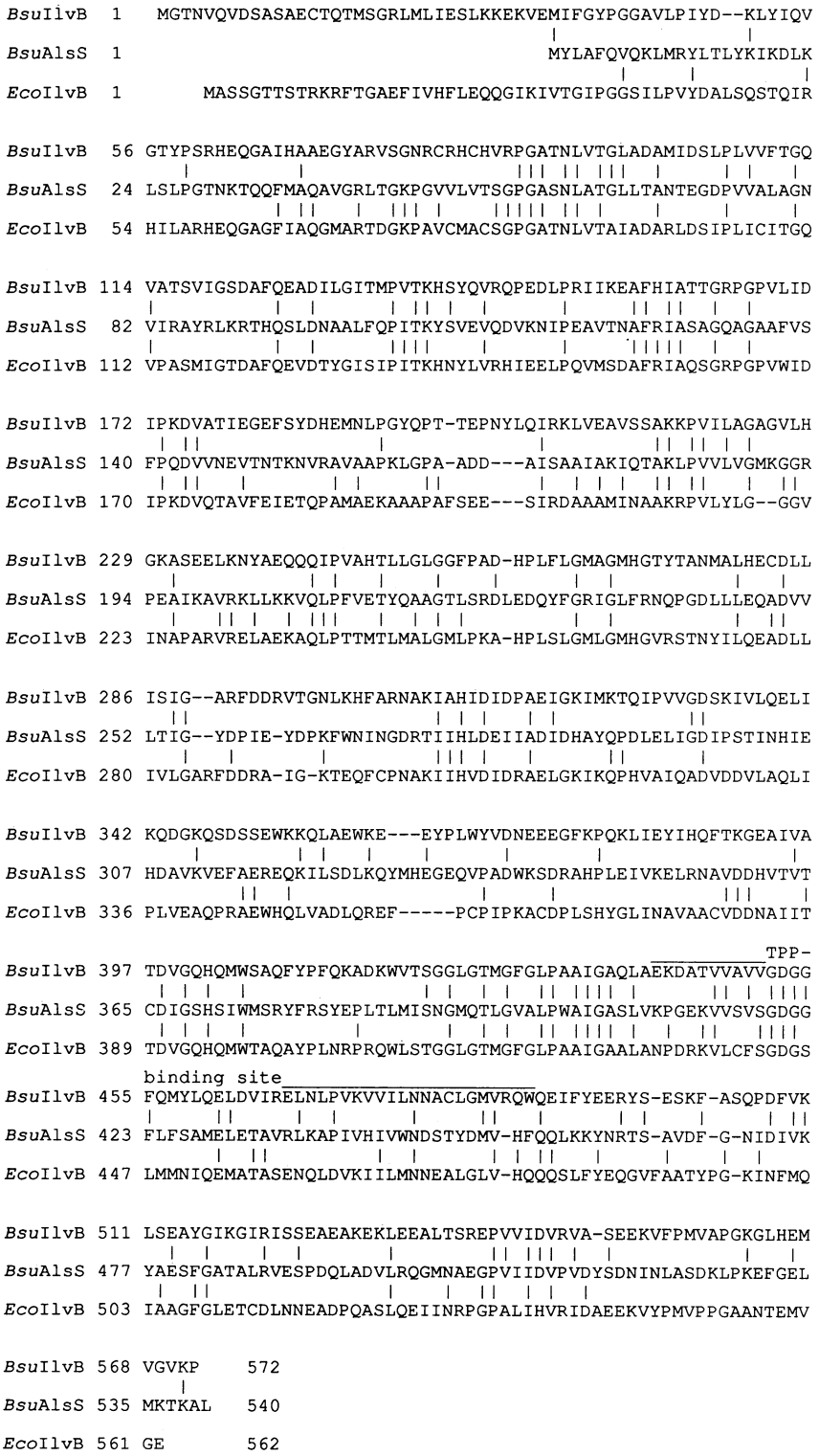


FIG. 5. Alignment of the AlsS protein of *B. subtilis* (this work), the IlvB protein of *B. subtilis* (28), and the IlvB protein of *E. coli* (35). Lines indicate amino acid identity. Dashes represent gaps introduced to maximize sequence alignments. A putative thiamine PP_i (TPP)-binding site (8) is indicated.

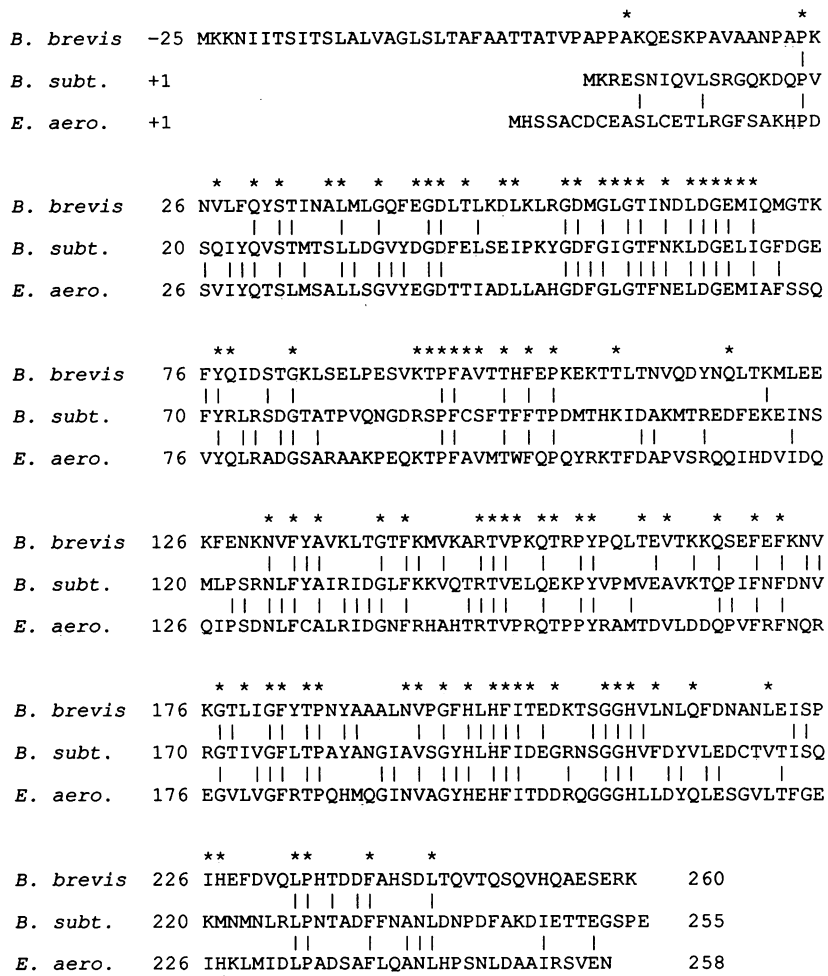


FIG. 6. Sequence alignments of AldC of *E. aerogenes* (25), AldC of *B. brevis* (2), and AlsD of *B. subtilis* (this work). Lines indicate identical amino acids. Asterisks above the alignments indicate amino acid identities between the AldC proteins of *E. aerogenes* and *B. brevis*. The sequence shown for the *B. brevis* enzyme is that of the unprocessed enzyme; the N-terminal sequence of mature AldC produced by *B. brevis* is generated by cleavage at two predicted signal peptidase cleavage sites, followed by removal of an N-terminal alanine residue.

−10 sequences, respectively, with 17 bp between them. The absence of any additional transcripts from cultures grown with acetate suggests that the increase in transcription of the *alsS-lacZ* fusion in both wild-type and mutant *alsR* backgrounds induced by acetate (data not shown) is due to increased transcription from this promoter.

We have looked for DNA sequences in this region similar to those that bind such positive control proteins as the *E. coli* IlvY protein (34) and that have been found in the target DNAs of several of the LysR family proteins (1). We have found similar sequences (42) but have not yet shown any functional significance for them. We have overexpressed the AlsR protein and have demonstrated that it binds specifically to a 309-bp fragment that contains the intergenic sequence and extends into the coding regions of both *alsS* and *alsR* (data not shown).

Nucleotide sequences of the *alsR1* and *alsR8* alleles. *alsR1* was shown to reside on a 2.3-kb *Hin*dIII fragment of λF cloned into pUC19 (plasmid pMR115), and the *alsR8* mutation was located on the same fragment in pMR221. Strain CU4800 (*ilvBΔ1 amyE::alsS-lacZ*), when transformed with linearized pMR115 or pMR221, generated Val^+ transformants that showed increased expression of the *lacZ* fusion at the *amy* locus. This suggested that the *alsR* mutations were *trans* acting and therefore not in the promoter of *alsS* but probably were in the *alsR* gene. Sequencing of the *alsR* alleles on pMR215 (*alsR*$^+$), pMR115 (*alsR1*), and pMR221 (*alsR8*) revealed single nucleotide changes in the ORFs that resulted in amino acid substitutions. In *alsR1*, a T-to-C transition at nucleotide 1128 resulted in the replacement of a threonine residue at position 201 in AlsR by an alanine residue. In *alsR8*, a T-to-G transversion at nucleotide 929 resulted in replacement of a serine residue at position 234 in AlsR by an alanine residue.

Disruption of *alsS* and *alsR* and their phenotypes in *B. subtilis*. Plasmids pMR100S and pMR100R, carrying disrupted copies of *alsS* and *alsR*, respectively, were linearized with *Eco*RI and introduced by transformation into competent cells of CU371 (*ilvBΔ1 alsR*$^+$). Chloramphenicol-resistant transformants were selected and demonstrated to have disrupted copies of the *alsS* and *alsR* genes in place of the wild-type alleles. Purified transformants were then patched onto minimal medium with various additives. The results of this experiment are shown in Table 2. Loss of AlsS in an *ilvBΔ1* background results in an absolute requirement for isoleucine and valine for growth on minimal medium. Inac-

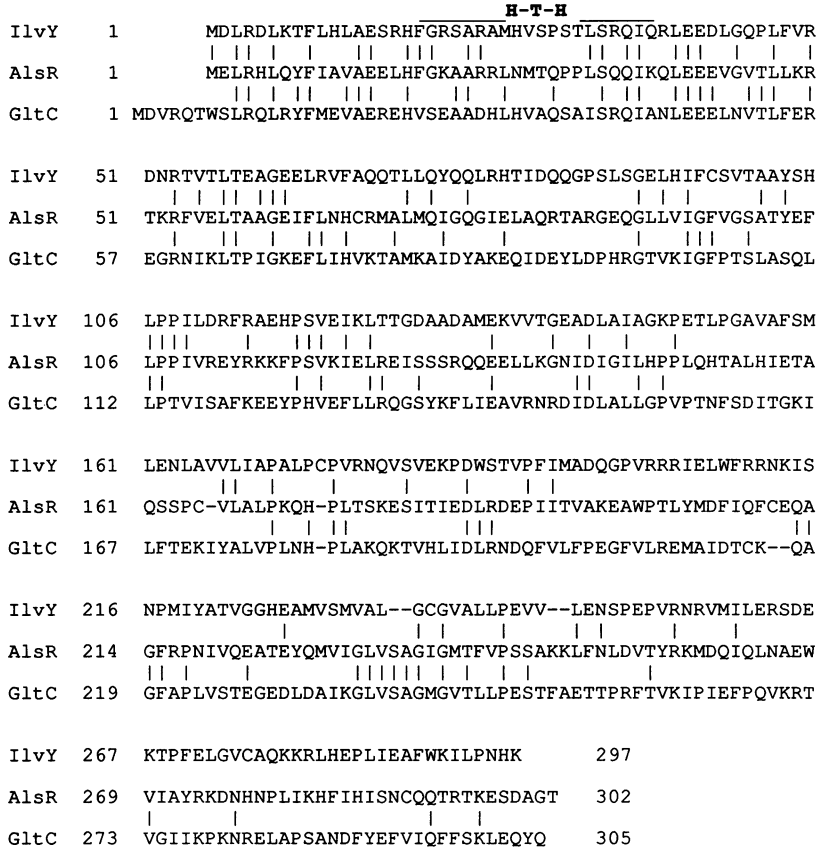


FIG. 7. Alignment of the amino acid sequences of IlvY of *E. coli* (33), GltC of *B. subtilis* (1), and AlsR of *B. subtilis* (this work). Dashes represent gaps introduced to maximize sequence similarities. Lines indicate amino acid identity. AlsR and IlvY are 22% identical; AlsR and GltC are 26% identical. H-T-H, helix-turn-helix motif.

tivation of *alsR* prevents expression of *alsS*; the cells require the addition of valine as well as isoleucine for growth on minimal medium. This strongly suggested that AlsR is a positive activator of *alsS*. The requirement for valine is not alleviated by the addition of potassium acetate to the medium, indicating that the induction of AlsS by potassium acetate is mediated at least in part by AlsR.

Disruption of *alsD* and its phenotype in *B. subtilis*. Plasmid pMR122, carrying a *cat* gene within *alsD*, was linearized with *Sca*I and introduced into competent cells of CU4768 and CU371, selecting for chloramphenicol resistance. Transformants were purified on chloramphenicol plates, tested to ensure disruption of the *alsD* gene (see Materials and Methods), and then tested for acetoin production, as measured by the Voges-Proskauer test. They were also tested for their ability to grow on minimal medium-isoleucine plates, to determine whether the disruption of *alsD* had any effect on their growth rates due to increased valine production. No change in growth rate on minimal medium-isoleucine plates was detected. In addition, no difference in growth rate was detected when otherwise isogenic *alsR1* strains were grown in liquid minimal medium containing isoleucine (data not shown).

In the Voges-Proskauer test, CU4768 (*ilvBΔ1 alsR1*) produced detectable amounts of acetoin during the exponential growth phase, as expected for a strain that carries the *alsR1* allele. However, an *alsR1* strain that also carried the disrupted allele of *alsD* (CU4390) did not produce acetoin in the exponential phase; nor did CU371 (*ilvBΔ1 alsR*$^+$). Similar results were obtained for cells harvested 30 or 60 min into the stationary phase; CU4390 produced a greatly reduced amount of acetoin, less than that seen for CU371 (Table 3). After 4 h in the stationary phase, all strains produced a significant amount of acetoin. It should be noted that acetolactate is also spontaneously broken down, resulting in a positive test.

Regulation of transcription of the *alsSD* operon. Plasmid pMR106 (*alsS-lacZ*) was linearized with *Sca*I and introduced into the chromosome of various strains of *B. subtilis*. These strains were assayed for β-galactosidase activity in the exponential and stationary phases. The results of these assays are shown in Fig. 9. In a wild-type strain of *B. subtilis* (CU4800) carrying the *alsS-lacZ* fusion integrated at the *amyE* locus, *alsS* transcription paralleled what has been seen for enzyme activity; it was induced in the stationary phase. The increase in transcription seen in the stationary phase was about 10-fold.

In an *alsR1* background there was an increase in the amount of transcription of *alsS* compared with that seen in the wild type. This increase was seen in both the growth and stationary phases and was approximately three- to fivefold, depending on the individual experiment.

In an *alsR*::*cat* background, expression of the *alsS-lacZ* fusion was similar to wild-type expression during growth, but no increase in transcription was seen when the cells

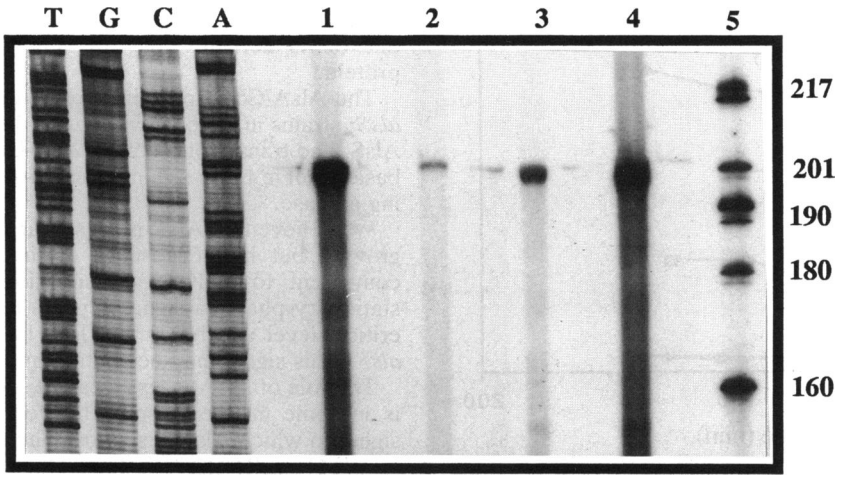


FIG. 8. Results of a primer extension experiment. Reactions were carried out as described in Materials and Methods. RNA was isolated from CU4955 grown in minimal medium containing isoleucine and valine. Lane 1, RNA isolated from stationary-phase (1 h after the end of log-phase growth) cultures without 40 mM potassium acetate; lane 2, RNA isolated from stationary-phase cultures with potassium acetate; lane 3, RNA isolated from exponential-phase cultures without potassium acetate; lane 4, RNA isolated from exponential cultures with potassium acetate; lane 5, pBR322 cut with *Msp*I as a size standard (sizes are shown in base pairs). The sequence ladder (lanes T, G, C, and A) was generated with primer 106.

entered the stationary phase; expression remained at the low level (2 to 3 Miller units) seen during growth.

We have also examined the transcription of *alsS* in an *alsA1* mutant background. In strains of *B. subtilis* carrying the *alsA1* mutation (41) AlsS activity is reduced to the point that *ilvBΔ1 alsR1 alsA1* strains do not grow on minimal medium unless isoleucine and valine are added (Table 2). The transcription of *alsS* in *alsA1 alsR1* cultures grown with glucose parallels that seen for *alsR*::*cat* strains.

Plasmid pDH32, containing a promoterless *lacZ* gene, was introduced into the *amyE* locus of CU371, and β-galactosidase activity was measured in the exponential and stationary phases. The background β-galactosidase activity was determined to be less than 1 Miller unit.

DISCUSSION

The genes required for the production of acetoin, *alsS* and *alsD*, are located at 314° on the chromosomal map (41) and have been cloned and sequenced. A third gene required for the formation of acetoin, *alsR*, was also cloned and sequenced.

TABLE 2. Phenotypes of *alsS*, *alsR*, *alsA*, and *alsD* mutants

Strain (genotype[a])	Growth[b] on plates of minimal medium + Ile		
	Alone	+40 mM potassium acetate	+Val
CU371	−	+	+
CU532 (*alsR1 alsA1*)	−	−	+
CU845 (*alsR1 hisA1*)[c]	+	+	+
CU4769 (*alsR*::*cat*)	−	−	+
CU4774 (*alsS*::*cat*)	−	−	+
CU4930 (*alsR1 alsD*::*cat*)	+	+	+

[a] All strains are *ilvBΔ1*.
[b] +, growth; −, no growth.
[c] Histidine was added to plates.

It has been proposed that the formation of acetoin and its reduced product butanediol plays two important roles in the cell. One is the maintenance of intracellular pH (4, 27). In addition, reduction of acetoin to generate butanediol may replenish pools of NAD^+ (4), although the gene encoding the enzyme that carries out this reaction has not been found in *B. subtilis*.

The *alsS* gene encodes a protein homologous to the IlvB protein of *B. subtilis* and to the AHAS isozymes of *E. coli*. When *alsS* is disrupted, an *ilvBΔ1 alsR1* strain is no longer able to grow on minimal medium lacking valine. This is very strong evidence that this gene encodes the catabolic acetolactate synthase.

The *alsD* gene encodes a protein with strong similarity to the known acetolactate decarboxylases of *E. aerogenes* and *B. brevis*. When *alsD* is disrupted, cells produce acetoin in the stationary phase at a delayed and reduced rate. The weakly positive results seen may in part be due to spontaneous breakdown of acetolactate. There does not appear to be any change in the growth rate of strains lacking *alsD* on minimal medium containing isoleucine but lacking valine.

There is no indication of a signal sequence preceding the AlsD protein of *B. subtilis*. The AlsD protein therefore does not appear to be an exoenzyme, unlike the *B. brevis* enzyme (2).

TABLE 3. Acetoin production by *alsD*$^+$ and *alsD* mutant strains of *B. subtilis*

Strain	Genotype[a]	Acetoin production[b] at:		
		Log phase	t = 60 min	t = 240 min
CU371	*alsR*$^+$	−	+	++
CU4768	*alsR1*	++	++	++
CU4930	*alsR1 alsD*::*cat*	−	±	++

[a] All strains are *ilvBΔ1*.
[b] Measured by the Voges-Proskauer test. t, length of time into the stationary phase; −, no acetoin production; ±, very weak acetoin production; +, moderate acetoin production; ++, strong positive acetoin test.

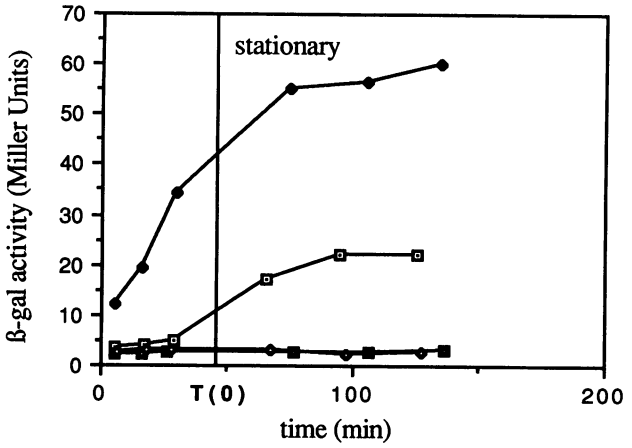


FIG. 9. Transcription profile of the *alsS-lacZ* fusion in various genetic backgrounds. Cultures were grown in LB broth plus 0.5% glucose, and samples were collected at various times in the log and stationary phases. Open squares, wild-type (*alsR*$^+$ *alsA*$^+$) strain (CU4800); closed diamonds, an *alsR1 alsA*$^+$ strain (CU4801); open diamonds, an *alsR*::*cat* strain (CU4810); closed squares, an *alsA1 alsR1* strain (CU4804). T(0) stands for the end of the exponential phase.

alsSD expression is limited to the stationary phase, and mutations in at least two genes (*alsR* and *alsA/ccpA*) have been shown to affect the expression of *alsS*. The regulation of *alsS* is controlled at least in part at the level of transcription. During growth, *alsS-lacZ* transcription occurs at a very low level in wild-type cells. Transcription increases when the cells enter the stationary phase.

The divergently transcribed *alsR* gene encodes a protein belonging to the very large LysR family of transcriptional regulators, which includes the GltC protein of *B. subtilis*. Most of these proteins are positive regulators, and most are activated by inducing ligands. Many have been shown to be autogenously regulated (1, 12, 26, 30). Where it has been studied, these proteins bind to their DNA-binding sites in either the presence or absence of the inducer (30, 34).

A number of mutations in *alsR* that result in an increase in the expression of the *alsS-lacZ* fusion and in increased synthesis of AlsS over that seen in the wild type during all stages of growth have been isolated (unpublished data). *alsR* has been disrupted and shown to be required for the growth of *ilvBΔ1* strains on minimal medium plus isoleucine in the absence of valine. Strains lacking AlsR show only very low levels of transcription of an *alsS-lacZ* fusion. This is strong evidence that AlsR is indeed a transcriptional activator of *alsS*.

The transcription of the *alsS-lacZ* fusion seen in strains lacking *alsR* is at the same low level seen in wild-type cells during logarithmic growth; thus, the role of AlsR seems to be limited to increasing the transcription of *alsS* in response to a signal that appears or accumulates in the stationary phase. The *alsR1* mutation results in increased transcription through the *alsSD* operon during both the exponential and stationary phases. Thus, the mutant AlsR protein still appears to be responsive to its putative signal. Transcription of the *alsS-lacZ* fusion in both the *alsR*$^+$ and *alsR1* backgrounds is increased in response to added acetate during all stages of growth. AlsS activity in strains lacking AlsR is no longer inducible by acetate, suggesting that acetate induction may occur through AlsR. It is possible that AlsR activity is influenced by changes in intracellular pH or that acetate or one of its derivatives is an inducing ligand of the AlsR protein.

The AlsA/CcpA protein is required for the expression of *alsS*; strains in which *alsA/ccpA* is disrupted do not produce AlsS, and transcription of the *alsS-lacZ* fusion is reduced to basal level in these strains when grown on medium containing glucose.

We believe that AlsR is present in the cells at all stages of growth, but in the absence of inducing ligand, it is not competent to activate transcription. Upon entry to the stationary phase, a signal appears or accumulates to some critical level and binds to AlsR, initiating transcription of *alsS*. This signal may be acetate or a related molecule.

The role of AlsA is less clear. It seems unlikely that AlsA is in some manner responsible for the production of the signal to which AlsR responds. The transcription of *alsS* in an *alsA1 alsR1* strain is lower than that seen in an *alsR1* strain in the exponential phase. AlsR1 appears to be able to activate transcription of *alsS* in the absence of its inducer, and so we would expect transcription of *alsS* in an *alsA1 alsR1* strain to be at the level seen during the exponential phase in the *alsR1* strain.

Alternatively, AlsA may play a more direct role in the regulation of *alsS* by binding to a sequence upstream of *alsS*. AlsA, homologous to LacI and GalR repressors of *E. coli*, has been shown to be required for glucose repression of the α-amylase gene (10). A sequence within the *amyE* promoter similar to *lacO* of *E. coli* has been shown to be the site of mutations that affect glucose repression of *amyE* (22, 32), but it is not yet known whether AlsA interacts with this site. There is no site obviously similar to *amyO* in the sequences upstream of *alsS*; it is therefore unknown whether AlsA binds to sequences upstream of *alsS*.

ACKNOWLEDGMENTS

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